The following publications show that antibodies to Ep-CAM were per se known and available to those skilled in the art, though none of the publications describe the presently claimed active immunization therapy.

PUBLICATION	DISCLOSES
1. Ross et al	MAbs GA733 and 17-1A detect the same antigen, though perhaps a different epitope.
2. Sears et al (1984)	MAb 17-1A was known and available to those skilled in the art.
3. Sears et al (1985)	Similar to Sears `984
4. Samonigg et al	Antibody 73-3 reacts with same antigen as MAb17-1A.
5. Hayden et al	Antibodies against Ep-CAM could be made by skilled person by genetic engineering techniques.
6. Cirulli et al	Antibody KS1/4 is an anti-Ep-CAM Mab.
7. Strnad e al	Amino acid and DNA sequence of KSA (identical to Ep-CAM) was known so one skilled in the art could make anti-Ep-CAM antibodies.
8. Herlyn et al	MAb 17-1A was known and available to those skilled in the art.
9. USP 6,444,207 (Centocor)	MAb 17-1A was know to be used in high doses.

EXHIBIT 2

Vol. 135, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
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ISOLATION AND CHARACTERIZATION OF A CARCINOMA-ASSOCIATED ANTIGEN

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Summary. GA733 is a murine IgG_{2a} monoclonal antibody (MAb) against human gastric carcinoma and is highly tumoricidal in nude mice. The GA733 antigen is a cell surface protein with two subunits of 30,000 and 40,000 daltons. The antigen isolated by immunoaffinity chromatography consists mainly of the 30,000-dalton subunit which bears the GA733 epitope. This subunit displayed several isoelectric points between 6.9 and 7.7. Anti-colon carcinoma MAb 17-1A also detects this antigen, but probably binds to a different epitope.

We have demonstrated that murine IgG_{2a} monoclonal antibodies (MAbs) have a potent tumoricidal effect in nude mice (1). As a result of these studies, therapeutic trials were begun in humans using the IgG_{2a} anti-colorectal carcinoma MAb 17-1A (2). Beneficial effects of this treatment might be due to the activation of human effector cells or to the induction of anti-idiotypes and anti-anti-idiotypes, thereby producing a long-term immune response to the tumor (3-4). Recently, the trials have been extended to include IgG_{2a} MAb GA733, which detects a glycoprotein with 30,000- and 40,000-dalton subunits (5). In this study we have isolated and biochemically characterized the GA733 antigen. We find that MAbs GA733 and 17-1A detect the same protein although, based on competition studies and anti-idiotype studies, they most likely bind to different epitopes.

MATERIALS AND METHODS

Cell lines and tumors. Colorectal carcinoma cell line SW948 was grown in culture using Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum. For growth of SW948 tumors, 5 X 10^6 cells were injected subcutaneously into nude mice. The resulting tumors (5-20 mm) were excised about 8 weeks later and stored at -70°C.

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MAbs and immunoaffinity matrices. The properties of 17-1A and GA733 (IgG_{2a}) MAbs have been described (5-6). Culture supernatant from hybridoma P3X63Ag8 which secretes MAb MOPC21 (IgG_1) was used as a nonspecific control. In some cases BR15-6A MAb (IgG_{2a}, anti-Y carbohydrate determinant) was used as a negative control. IgG was purified from ascites fluid by protein A Sepharose chromatography. MAbs dissolved in 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, were coupled to cyanogen bromide-activated Sepharose at 4°C.

Isolation of GA733 antigen. The tumors were thawed, minced into fine pieces, and extruded through a wire screen. As judged by trypan blue staining, this preparation contained cell fragments and no intact cells. The preparation was centrifuged at 105,000xg for 60 min and the cell fragments in the pellet were extracted with 5 volumes of NP-40 buffer (0.5% NP-40, 140 mM NaCl, 10 mM NaF, 10 mM Tris, 5 mM EDTA, 100 Kallikrein IU/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, pH 7.5) for 16 hr. The extract was clarified by centrifugation at 105,000xg for 60 min and filtered through a 0.45-μm filter.

The extract from about 15 grams of SW948 tumors was passed through a bovine serum albumin (BSA)-Sepharose column (3 ml of 10 mg/ml) equilibrated with 0.2% NP-40, 20 mM Tris, pH 7.5. The extract was then passed twice through a GA733-IgG column (2 ml of 5 mg/ml). The column was then washed with 40 ml of the same buffer and eluted with 0.2% NP-40, 50 mM diethylamine, pH 11.0. The receiving tube for each 8 ml fraction contained 0.1 ml, 1 M Tris, pH 6.0. The purified protein was stored at -20°C.

Immunoprecipitation, gel electrophoresis, and Western blotting. Immunoprecipitations were carried out using agarose derivatized with goat antibody directed against mouse immunoglobulin (IgG) (7). One-dimensional gel electrophoresis was performed according to Laemmli (8). Two-dimensional gel equilibrium electrophoresis was by the method of O'Farrell (9) using pH 3.5-10 ampholines. Western blotting was performed according to Towbin et al. (10).

Radioiodination of GA733 antigen. The antigen was radioiodinated on ice with 5 mM chloramine-T using 0.4 mCi $Na^{125}I$ per sample. After 2 min the reaction was stopped with 14 mM sodium metabisulfite and then applied to a PD-10 column equilibrated with 0.1% BSA, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4. The fractions at the excluded volume of the column were pooled and stored at -20°C.

Binding of GA733 antigen to bound antibody. Solid-phase microtiter plates (Costar Plastics) were incubated with IgG (1 μg/well) in phosphate-buffered saline (PBS) (0.15 M NaCl, 10 mM sodium phosphate, 0.02% NaN_3, pH 7.5) for 16 hr at 4°C. Wells were blocked with PBS + 2% BSA for 3 hr at room temperature, and were washed with PBS + 2% gamma globulin-free horse serum. Radioiodinated GA733 antigen (25,000 cpm/well) and in some cases competing MAb were incubated at 4°C in the wells. After 16 hr, the wells were washed 3 times with the horse serum buffer and eluted with Laemmli sodium dodecyl sulfate (SDS) sample buffer for 30 min at 37°C. Samples were boiled, assayed for radioactivity in a gamma counter, and further analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Anti-idiotypic antibodies (anti-Ids). Anti-Ids were prepared against MAbs GA733 and 17-1A as described and were shown to react with the combining sites of the corresponding MAbs (11). These anti-Ids were used in direct binding assays with various MAbs as targets and in binding inhibition assays in which binding of MAb to either colon carcinoma target cells or homologous anti-Ids was inhibited by homologous or heterologous anti-Ids as described in detail elsewhere (11).

RESULTS AND DISCUSSION

Isolation and molecular characterization of GA733 antigen. Cell fragments from SW948 tumors were solubilized with NP-40 buffer, clarified by

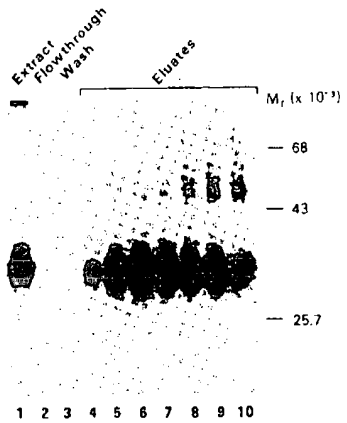

Figure 1. Analysis of GA733 immunoaffinity chromatography by Western blotting. Cell extract (10 ml) was applied to a GA733 IgG Sepharose column, washed with 40 ml of 0.2% NP-40, 20 mM Tris, pH 7.5, and eluted 8 times with 8 ml of 0.2% NP-40, 50 mM diethylamine, pH 11.0. Twenty-five µl of each fraction was mixed with SDS-PAGE sample buffer lacking mercaptoethanol, boiled and subjected to SDS-PAGE. The proteins present in the gel were electrophoretically transferred to nitrocellulose, and the GA733 antigen was detected with GA733 MAb and [^{125}I]goat anti-mouse IgG by the Western blot procedure.

ultracentrifugation, and applied to a GA733 immunoaffinity column. After thorough washing, the protein bound to the column was eluted with mild base. Analysis of the resulting fractions by Western blotting demonstrated that most of the antigen was bound by the column (Fig. 1) and was released from the column by the base. Binding of the GA733 MAb to antigen is probably dependent on the tertiary conformation of the protein since samples treated with mercaptoethanol gave no bands in Western blots.

The eluate was dialyzed against 0.1% SDS, 50 mM NH_4HCO_3 and lyophilized. This material was redissolved and iodinated with chloramine-T as described in Materials and Methods. The nonradioactive and the iodinated GA733 antigens were analyzed by SDS-PAGE and found to be nearly homogeneous by silver staining and autoradiography, respectively (Fig. 2A and B). Furthermore, the [^{125}I]GA733 antigen was immunoprecipitated by MAb GA733 but not by a control MAb, confirming that the purified protein is the GA733 protein. The [^{125}I]GA733 antigen was further characterized by two-dimensional gel

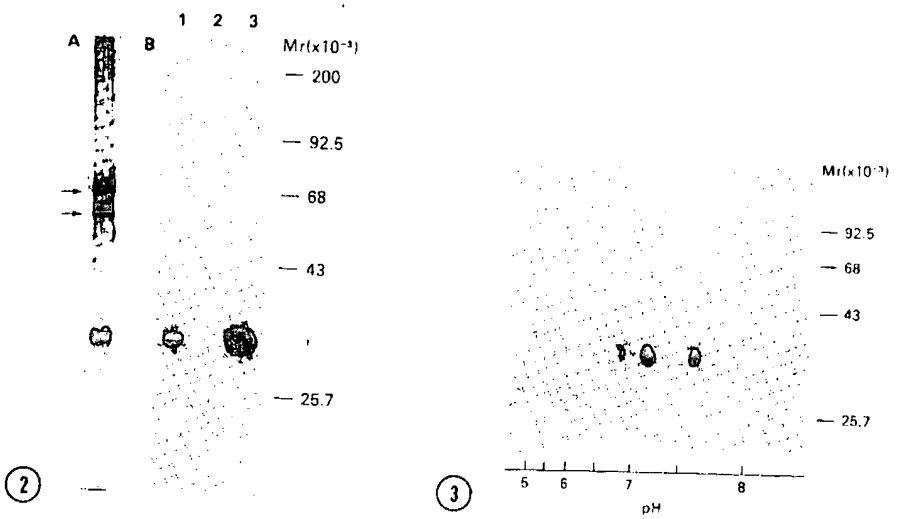

Figure 2. Analysis of purified GA733 antigen by SDS-PAGE. Panel A. Purified antigen was electrophoresed on a 10% polyacrylamide gel and detected by silver staining. Two nonspecific bands which appear even in lanes to which no sample was applied are marked with arrows. Panel B. [^{125}I] GA733 antigen was either analyzed directly by SDS-PAGE (lane 3) or first immunoprecipitated with GA733 MAb (lane 1) or with P3X63Ag8 (lane 2), a nonspecific control.

Figure 3. Charge heterogeneity of GA733 antigen. Radioiodinated GA733 antigen was analyzed by two-dimensional gel electrophoresis using ampholines in the pH 3.0-10.0 range.

electrophoresis (Fig. 3). Three major species were detected with pIs between 6.9 and 7.7

Binding of GA733 antigen by MAb 17-1A. GA733 MAb completely inhibited the binding of [^{125}I]17-1A MAb to SW948 cells while 17-1A MAb partially inhibited [^{125}I]GA733 MAb binding (not shown). To determine whether these MAbs detect the same antigen, microtiter plates were coated with either GA733, 17-1A, ME75-29, or BR15-6A IgG, the wells were blocked and incubated with [^{125}I]GA733 antigen, and washed. Bound radiolabeled protein was eluted with Laemmli sample buffer and analyzed by SDS-PAGE and autoradiography. Fig. 4A shows that both GA733 and 17-1A MAbs but not ME75-29 or BR15-6A bound [^{125}I]GA733 antigen. Between 5 and 15% of added cpm was bound by GA733 MAb, depending upon the batch of [^{125}I]GA733 antigen used. We then tested whether GA733 or 17-1A MAb would inhibit each other in binding to ^{125}I-labeled puri-

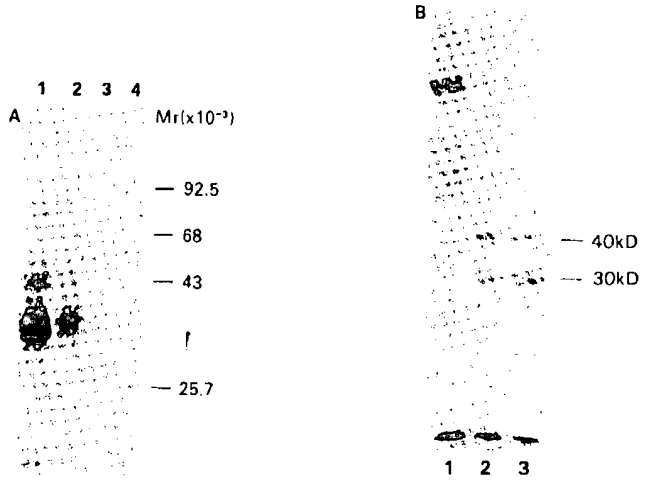

Figure 4. Binding of [^{125}I]GA733 antigen to immobilized MAbs. Panel A. Purified radioiodinated antigen bound to a microtiter plate derivatized with GA733 IgG (lane 1), 17-1A IgG (lane 2), ME75-29 IgG (lane 3), or BR15-6A IgG (lane 4) was analyzed by SDS-PAGE. Panel B. Proteins from extract of surface-labeled SW948 cells bound to microtiter plate derivatized with BR15-6A IgG (lane 1), 17-1A IgG (lane 2), or GA733 IgG (lane 3).

fied antigen. Fig. 5 shows that soluble GA733 MAb completely inhibited binding of [^{125}I]GA733 antigen by GA733 or 17-1A MAb bound to the plate. Soluble 17-1A MAb partially inhibited binding of antigen by GA733 or 17-1A MAb bound to the plate. Unrelated MAb BR15-6A did not significantly inhibit binding.

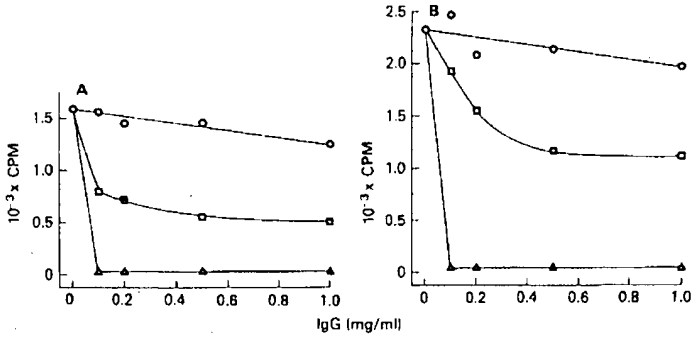

Figure 5. Inhibition by soluble IgG of binding of immobilized MAbs to [^{125}I]GA733 antigen. Antigen bound to an microtiter plate derivatized with either 17-1A IgG (A) or GA733 IgG (B) in the presence of soluble BR15-6A IgG (○), GA733 IgG (△), or 17-1A IgG (□) was quantitated by gamma counting.

Total cell extract of lactoperoxidase-surface radioiodinated SW948 cells was incubated in MAb-coated microtiter wells, washed, and eluted as above. MAbs GA733 and 17-1A bound both the 30,000- and 40,000-dalton forms of the antigen (Fig. 4B).

Idiotypic analysis of MAbs 17-1A and GA733. By Id analysis (11), no significant cross-reactions between the idiotypes of MAbs GA733 and 17-1A were detected.

Conclusions. We have isolated the GA733 antigen from SW948 tumor cells. The antigenic site is located on a 30,000-dalton protein with a pI of 6.9-7.7 and is sensitive to reduction of disulfide bonds. This purified protein will be useful for more detailed biochemical and sequence analysis as well as for the characterization of anti-Id and anti-anti-Id immune responses in cancer patients treated with MAb GA733 (4, 11, 13). These experiments also raise two questions that require further study. First, the antigen consists of two subunits with apparent molecular weights of 30,000 and 40,000 (5); however, the isolation procedure gave preparations with little or no 40,000-dalton species suggesting that this antigen form is lost during purification of the 30,000-dalton form or that the 30,000-dalton form is a breakdown product that readily forms during purification. Second, both anti-carcinoma MAbs GA733 and 17-1A bind to the GA733 antigen. It is likely that they bind to different but adjacent sites on the protein since they partially compete with each other for binding to the antigen and do not share idiotopes. However, the partial inhibition of GA733 MAb binding by 17-1A MAb may also reflect the 10-fold lower affinity of the 17-1A MAb (12). A detailed mapping of the antigen-antibody binding sites should aid in answering these questions.

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Effects of Monoclonal Antibody Immunotherapy on Patients with Gastrointestinal Adenocarcinoma

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Summary: Twenty patients with metastasis of gastrointestinal malignancies were treated with an anti-colorectal cancer mouse monoclonal antibody 1083-17-1A of the IgG_{2a} class between December 1980 and January 1983. With two exceptions, all patients received a single injection of monoclonal antibody in a dose range of 15–1,000 mg/patient. No untoward immediate or delayed reaction to the initial injection was observed in any of the patients. Mouse immunoglobulin circulated in the patients' blood for 2–50 days, depending on the dose of monoclonal antibody injected, and was detected in tumor tissue within 1 week of its administration. Eight of nine patients who received doses of 366–1,000 mg monoclonal antibody did not develop anti-mouse antibodies, while eight of nine who received less than 200 mg developed anti-mouse immunoglobulin antibody. Three of this heterogeneous group of patients have no detectable disease now—10, 13, and 22 months since immunotherapy. **Key Words:** Adenocarcinoma of colon and rectum—Immunotherapy—Monoclonal antibody.

We have previously demonstrated that it is safe to give an anti-colorectal cancer (CRC) mouse monoclonal antibody (MAb) 1083-17-1A (1), which specifically binds to (2A) and inhibits the growth of human gastrointestinal cancer in athymic mice (2,3), to patients with advanced gastrointestinal malignancies. This antibody does not bind to a wide variety of cultured cell lines from human and malignant tissues, or circulating blood cells, or to freshly resected colonic mucosa from patients whose tumors do bind antibody (2A). A single intravenous dose of this γ-2a mouse immunoglobulin was well tolerated by patients, and no symptoms of immediate allergy or delayed serum sickness were observed. As the antigen recognized by this antibody is bound to the tumor cell surface, and does not shed from the cell in culture nor into the sera of patients with advanced colon cancer,

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complexing of administered antibody in the circulation does not occur. $F(ab')_2$ fragments of this antibody conjugated to ^{131}I have been used to localize hepatic metastases of patients with CRC (4–6), and have been readministered without complications to some patients. One patient who was exposed repeatedly to intact antibody did have an adverse vasomotor and bronchospastic reaction (1).

Any treatment regimen with murine MAb requires an understanding and control of the human anti-mouse immunoglobulin antibody response, as well as knowledge of the duration of mouse immunoglobulin in the patient's circulation. Therefore, this study describes patterns of circulation of mouse immunoglobulin and development of the human anti-mouse immunoglobulin antibody response in patients receiving varying doses of mouse MAb (7) and, in some cases, chemotherapy or radiation therapy for their tumors. This report describes the 20 patients we have treated with MAb. Preliminary results on 4 of the 20 were published previously (1).

MATERIALS AND METHODS

Patient Selection

Twenty patients with biopsy-proven advanced gastrointestinal adenocarcinoma volunteered for inclusion in this experimental protocol for specific immunotherapy with MAb.

Patients were eligible for this study if their locally advanced tumor or its metastasis caused some complications that required a planned abdominal operative procedure, and if complete tumor resection for cure was not feasible. Since the initial trials to demonstrate antibody binding on metastases by the immunoperoxidase techniques were unsuccessful, obtaining tissues for these specificity binding studies after *in vivo* injections was discontinued. Therefore, after 10 patients were so treated, the study was changed and an operative procedure was no longer required for eligibility. The study sample consisted of 15 men and 5 women, ranging in age from 40 to 80 years. Primary tumors were colonic (in 13 patients), rectal (in 4), gastric (in 2), and pancreatic (in 1). Ten patients received chemotherapy of some sort and two received radiation therapy (within 2 months of receiving antibody). Advanced disease was systemic to the liver and lungs in 11 patients; local only to the pelvis, peritoneum, or retroperitoneum in 3 patients; and both local and systemic in 6 patients.

The patient's complete blood count, bilirubin, serum creatinine and transaminase, alkaline phosphatase, and carcinoembryonic antigen (CEA) were obtained prior to entry into the study. Metastatic or locally invasive tumors were localized with contrast X-ray films, ultrasonographic studies, isotopic scans, computed axial tomography (CAT), and scanning by MAb 1083-17-1A $F(ab')_2$ fragments conjugated to ^{131}I (3 patients) (5). Patients were not required to have measurable lesions as detected by these techniques. Two patients were surgically explored for CAT scan–identified abnormalities that subsequently proved to be benign, but these patients had metastatic disease elsewhere that was not roentgenographically detected (see Table 1, patients 3 and 9).

TABLE 1. *Immunotherapy with 17-1A monoclonal antibody (MAb)*

Patient	Sex	Primary site	Amount of MAb received (mg)	Current status	Human anti-mouse antibody
1	M	R	15	AWD	+
2	F	C	150	D	−
3	M	C	150	D	+
4	M	G	200	D[a]	+
5	F	C	100	D	+
6	M	G	72	D	NT
7	F	R	128	NED	+
8	M	R	92	AWD	+
9	M	C	133	AWD	+
10	M	C	135	D	?
11	M	C	137	D	+
12	M	C	366	AWD[a]	−
13	F	C	440	AWD	−
14	M	P	433	NED	−
15	M	C	400	D	−
16	M	R	380	D	−
17	M	C	675	D	+
18	F	C	391	AWD	−
19	M	C	1,000	D	−
20	M	C	611	NED	−

[a] Received multiple doses of MAb.
AWD, alive with disease; NED, no evidence of disease; D, dead; R, rectum; C, colon; P, pancreas; NT, not tested.

Informed consent, describing this study and possible complications, was obtained from all patients, and the study was approved by the Clinical Investigation Review Committee of The Fox Chase Cancer Center.

Antibody

The preparation of murine MAb 1083-17-1A (IgG_{2a} isotype) against human CRC has been described previously (1). Briefly, ascites was collected aseptically, and centrifuged and filtered under sterile conditions through 0.22-μm Millipore filters. The filtrate was diluted with an equal volume of sterile 0.1 *M* Tris (pH 8.0) and 10 ml was applied to a sterile protein A–Sepharose column. This column was washed with 0.1 *M* Tris (pH 8.0) and the IgG_{2a} eluted with 0.1 *M* citrate (pH 4.5). After adjusting the pH to neutral, the eluate was dialyzed against saline. The immunoglobulin was 95% pure, as judged in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and was negative in the *Limulus* amoebocyte lysate assay at a concentration of 500 μg/ml. The concentration of mouse immunoglobulin added to the infusion volume varied from 1.3 to 20.2 mg/ml protein.

Antibody Administration

Each patient was treated in a Special Care Unit of The Fox Chase Cancer Center equipped to deal with possible untoward complications. An intravenous

line was maintained regardless of the route of antibody administration. Skin tests for sensitivity to mouse immunoglobulin were performed by scratching MAb into the dermis of the forearm and checking for a wheal or erythematous reaction. Skin tests for delayed hypersensitivity to common recall antigens were placed at the same time. The patient's vital signs, respiratory symptoms, and temperature were monitored during and for 3 h following MAb administration. The antibody was given intravenously in increasing dosages from 15 to 1,000 mg protein diluted in 250 ml normal saline. Antibody was given only once to all patients except two, one (no. 4) who received five separate injections over 11 days (1) and another (no. 12) who received a second injection of MAb 350 days after the first injection. Seventeen patients received MAb by slow (30–60 min) intravenous drip. In three patients who had only liver metastases, MAb was administered through a percutaneously placed hepatic artery catheter using a volumetric infusion pump. Antibody was given between 3 and 48 h prior to an operative procedure, if one was done.

Postantibody Monitoring

The patient's vital signs, temperature, and physical condition were closely observed during antibody infusion and for the next 24 h. Blood samples were obtained before treatment, hourly immediately after treatment, and subsequently every day for 10 days, at weekly intervals for 1 month, and then monthly for 3 months to monitor hepatic and renal function, as well as to test for the presence of circulating mouse immunoglobulin and human anti-mouse immunoglobulin response. The patients were then monitored for tumor response depending on the clinical presentation of their disease. In some cases, indicator lesions for direct evaluation of antibody response were removed or altered by operative intervention. Ten patients in this study had operative procedures after receiving antibody, and 11 have received chemotherapy or radiation after antibody administration.

Determination of Mouse IgG and Human Anti-Mouse Antibody in Blood of Patients

Immediately after injection of MAb, and at daily intervals until the 10th day, the serum of patients was assayed for the presence of mouse IgG. The test, which followed a procedure described before (1), consisted of exposing a 1:10 dilution of serum to rabbit anti-mouse IgG antibody and determining the binding by ^{125}I-labeled rabbit anti-mouse F(ab')$_2$ fragment of immunoglobulin. After the 10th day, the assays were made on serum samples obtained at less frequent intervals. The same serum samples were tested for the presence of human anti-mouse antibody. The procedure (1) consisted of incubating the patient's serum with mouse monoclonal anti-colon carcinoma antibody obtained from serum-free medium and detecting the binding by ^{125}I-labeled goat antibodies directed against F(ab')$_2$ fragments of human immunoglobulin.

RESULTS

No immediate allergic or other adverse effects were observed in the 20 patients (Table 1) with advanced gastrointestinal carcinoma who received one systemic intravenous or intraarterial dose of anti-CRC MAb. Increasing quantities of antibody from 15 to 1,000 mg were well tolerated. No changes in pulse, respiratory rate, or blood pressure were noted when intravenous flow rates were held below 250 ml/h. On three occasions, a flow rate greater than this led to a mild transient hypotensive response that was not associated with a change in pulse or respiratory rate and normalized upon slowing the administration rate. Two patients developed isolated pruritic urticarial lesions which resolved without treatment within 1 h; this occurred in patients who previously had had urticarial reactions in association with stress or anxiety. All patients' temperatures remained unchanged during and immediately after intravenous antibody administration. No respiratory symptoms were encountered during or after antibody administration, and no physical findings of bronchospasm or wheezing were noted. As described before (1), one patient who had repeated exposure to antibody (five injections over 11 days) developed a bronchospastic and vasoactive reaction on day 11 which was readily controlled. Retrospectively, it was determined that the results of the assay for antibody against murine immunoglobulin were elevated on day 10 (see Figs. 1 and 2 in ref. 1).

Hepatic, renal, and hematopoietic functions were unchanged in the immediate postimmunotherapy period and for 1 month thereafter. Those patients with previously altered hepatic or renal function because of their metastatic disease had unchanged or improved values of bilirubin, serum transaminase, and creatinine levels at 1 week to 1 month after antibody administration. Patients with no previous compromise of these functions showed unaltered values for the 3 months of follow-up. Total white blood cell count and differential counts remained the same. Changes in red blood cell count, hematocrit, and hemoglobin were consistent with operative procedures that were done on 10 patients and were not seen in other patients.

An erythematous rash on one patient's chest and abdomen was noted 12 days after immunotherapy, and spontaneously resolved in 2 days. Evaluation by a dermatopathologist confirmed this was related to the antibody administration. No other symptoms suggestive of serum sickness or delayed immunologic response were seen or reported for any patient in this study. Different routes of administration and previous treatment modalities did not alter the patients' tolerance to this form of immunotherapy. No gastrointestinal symptoms, save for mild diarrhea in two patients, were observed.

Eleven of the 20 patients had a large tumor burden and compromise of their renal or hepatic function when they received MAb; they have subsequently died or have progressive disease. Nine patients are alive 10–35 months after treatment. Three of these patients had hepatic metastases, two had pulmonary metastases, three had peritoneal or retroperitoneal recurrences, and two had local pelvic fascia infiltration of adenocarcinoma. The primary tumors in these patients were moderately well differentiated, except one, which was poorly differentiated.

TABLE 2. *Evaluation of immunotherapy with monoclonal antibody (MAb)*

Patient	Site of primary/ differentiation	Site of metastases	Therapy prior to MAb	Operation	Dosage MAb (mg)[a]	Therapy post-MAb	Months without evidence of tumors	CEA (ng/ml)
7	R/M	Lung, pelvic	Chemotherapy	Thoracotomy, ischiorectal biopsy	125	None	22	5 Pre-antibody, <2.5 since
14	P/M	Liver	Chemotherapy	None	433	None[b]	13	Never elevated
20	C/M	Peritoneal	None	Laparotomy	611	Laparotomy	10	NT

[a] Within 3–48 h before the operation.
[b] Chemotherapy discontinued after one injection.
R, rectum; C, colon; P, pancreas; M, moderately well differentiated; CEA, carcinoembryonic antigen; NT, not tested.

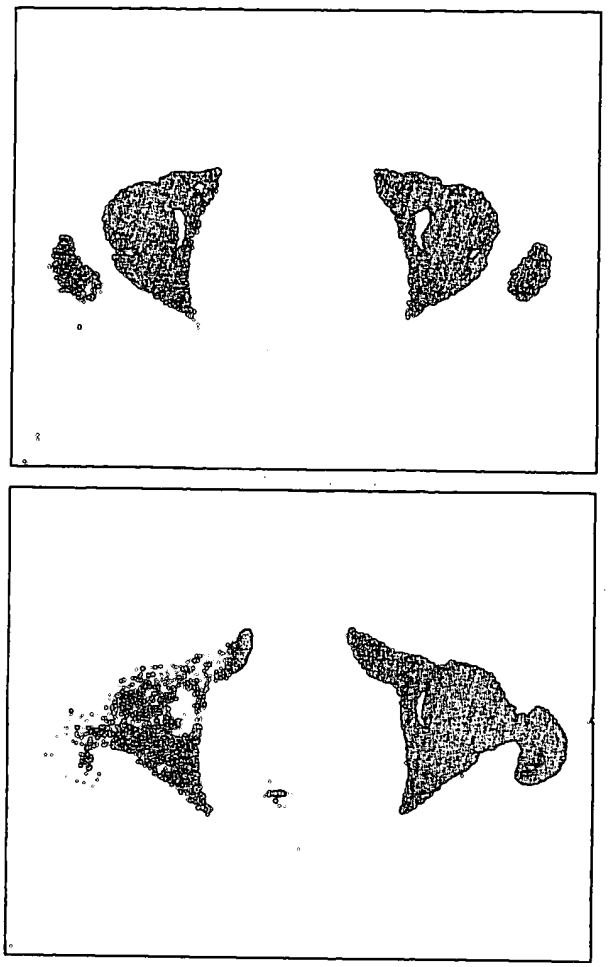

FIG. 1. Computed axial tomographic (CAT) scan of the pelvis made at the time of antibody administration. Two adjacent cuts through the lower pelvis demonstrated the cephalad portion of the retrorectal mass, which does not invade the rectal mucosa. Distal to this, a nodule of metastatic adenocarcinoma, the same as the previously resected rectal primary adenocarcinoma, was transrectally biopsied.

Three patients are currently tumor-free, 22, 13, and 10 months after MAb injection (Table 2). A brief summary of the clinical events of two of these patients follows.

Patient No. 7

A 57-year-old woman had a rectal cancer locally resected and wedge excision of pulmonary metastasis 6 months prior to receiving MAb. During the intervening period she received chemotherapy (5-FU and methyl-lomustine [CCNU]). An ischiorectal mass grew progressively despite chemotherapy. She was given 125 mg MAb on December 15, 1981, and the recurrent ischiorectal cancer was excisionally biopsied on December 16, 1981. Subsequently, the patient refused further chemotherapy. A CAT scan of the pelvis 1 week after MAb administration revealed a dense, soft tissue mass with early sacral erosion (Fig. 1). Ten months later, no recurrent tumor was detected in the presacral region by CAT scan (Fig.

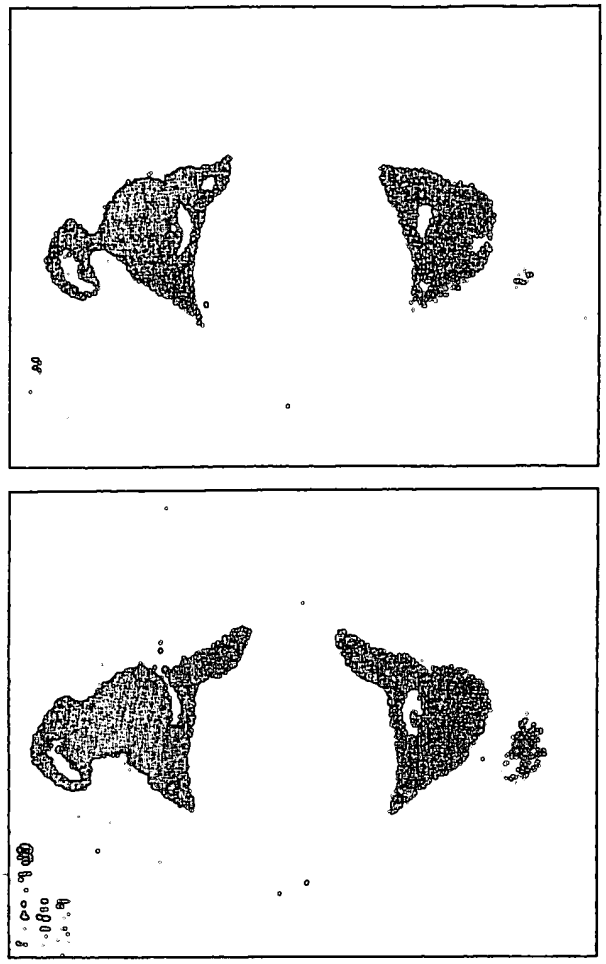

FIG. 2. Pelvic CAT scan. Two cuts made at the similar pelvic and acetabular landmarks taken 10 months after antibody administration (Fig. 1). Resolution of the mass has occurred, and the rectal wall is not distorted.

2). CEA level, which was 18 ng/ml prior to her initial resection and rose from <2.5 to 5 ng/ml during chemotherapy, has been <2.5 ng/ml since MAb treatment. She is currently well and her chest X-ray film remains clear.

Patient No. 14

A 52-year-old man had a metastatic pancreatic carcinoma biopsied while undergoing a choledochojejunostomy in July 1982. Postoperatively he was started on 5-FU and Adriamycin®. CAT scan showed progression of hepatic defects with retroperitoneal adenopathy, and he was given 433 mg MAb in autologous leukocyte-rich plasma in late September 1982. His CEA values were never elevated. In October 1982, he refused further chemotherapy. He currently feels well, and CAT scan shows progressive reduction of defects in the head of the pancreas and the liver.

Three other patients who received 15, 92, and 366 mg antibody and had no other therapy had no evidence of metastatic disease for 24, 9, and 12 months,

respectively, after immunotherapy. Subsequently, disease at the original metastatic site in the retroperitoneum, liver, or peritoneum was again documented by biopsy. Two of these three patients have since been treated by other conventional modalities, i.e., operative resection and radiation therapy, and one (no. 12) received, in addition, another injection of 150 mg 17-1A MAb (see below). None of these patients had any other therapy between the time they received MAb and the time the metastases were again documented. They are alive at present.

Presence of Mouse IgG in Patients' Serum

Nine patients were available over at least 10 months for detailed study of levels and duration of mouse IgG in their serum. High levels of mouse IgG were observed within 1–3 h of administration of MAb. Maintenance of these levels was related in general to the dose of MAb given, and was not influenced by the route of administration. A more rapid clearance of MAb was not detected in patients who had this administered into their hepatic arteries.

As shown in Fig. 3 (top), mouse IgG in blood of patients who received 15–133 mg MAb (nos. 1, 7, 8, and 9 in Table 1) circulated at detectable levels for 2–8 days, with the shortest duration following administration of the lowest (15 mg) dose of MAb.

In contrast, five of the six patients who received high doses (366–675 mg) of MAb circulated mouse IgG at much higher levels than in the preceding group and for considerably longer periods of time (Fig. 3, bottom). One of the patients, however, who received 366 mg MAb, ceased mouse IgG circulation on the 5th day after injection of MAb, even though he did not develop human anti-mouse antibody (see below).

Initial attempts to demonstrate antibody binding on tissues after systemic injection in these patients was impossible. Subsequently, techniques were developed to document antibody in resected tissues. One patient (no. 18) has had multiple biopsies of her skin metastasis or normal rectal mucosa prior to and after injection with MAb. These specimens were evaluated by modified fixation and immunoperoxidase techniques (8). Murine immunoglobulin could be detected on the metastatic tumor 48 h after injection. This was seen as focal staining at 1 week and was not evident 3 weeks after injection. Antibody did not bind to the dermal or epidermal elements. Positive but less intense staining was detected on the normal rectal mucosa at 48 h which was not detected thereafter. As these techniques were not developed until 18 months after the study was started and since they require fresh tissues, retrospective review of previously treated patients' lesions could not be accomplished.

Human Anti-Mouse Antibody Response

As shown in Table 3, eight of nine patients injected with a dose of MAb of $\leq$200 mg developed anti-mouse antibodies. The only patient who failed to produce these antibodies was anergic to recall antigens and was terminally ill at the times he was treated with MAb. Of nine patients who received larger (366–1,000 mg)

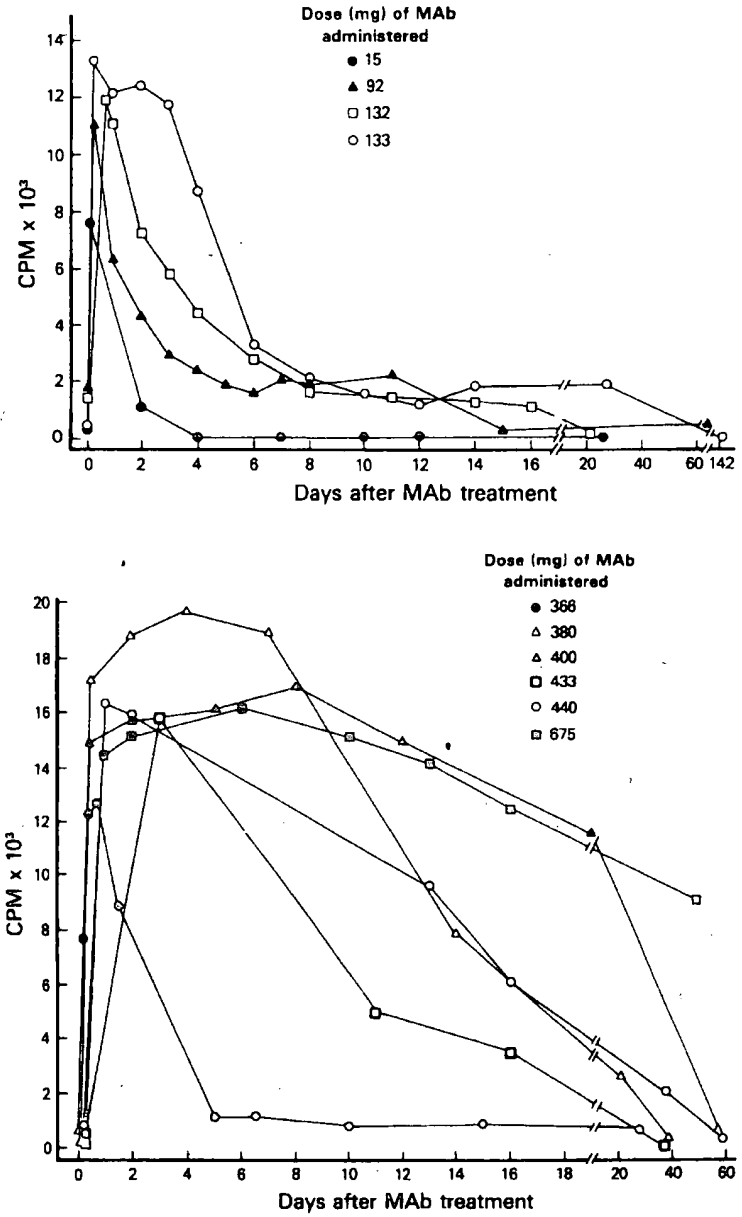

FIG. 3. Persistence of mouse IgG in sera of patients treated with monoclonal antibody (MAb). Patient's serum, diluted 1:10 in buffer, was exposed to rabbit anti-mouse IgG antibody and the binding was detected by ^{125}I-labeled rabbit anti-mouse F(ab')$_2$ immunoglobulin. **Top:** Data obtained from four individuals who received low or medium doses of MAb 1083-17-1A. **Bottom:** Data obtained from six individuals who received large doses of MAb 1083-17-1A.

TABLE 3. *Correlation between dosage of monoclonal antibody (MAb) and human anti-mouse antibody response*

Amount of mouse MAb given in single injection (mg)	Ratio of patients developing human anti-mouse antibody
15–200	8[a]/9
366–1,000	1[b]/9

[a] One immunosuppressed patient who received 150 mg MAb did not develop human anti-mouse antibodies.

[b] Patient who received 675 mg showed presence of human anti-mouse antibodies only between the 14th and 37th day after treatment.

doses of MAb, only one developed human anti-mouse antibodies transiently between 14 and 37 days after treatment. This patient had previously been skin-tested with an $F(ab')_2$ fragment of 17-1A for an imaging trial. When anti-mouse antibodies developed, response was first detected in the blood at 6–10 days after administration of MAb, and this coincided with the cessation of circulation of mouse IgG (see Fig. 3, top), with the exception of the patient who circulated mouse IgG for 5 days and did not develop human anti-mouse antibodies (see Fig. 3, bottom). The level of human anti-mouse antibody reached a peak at 14–21 days after administration of MAb, and thereafter the concentrations of those antibodies usually decreased to an undetectable level at 100 days after MAb administration.

Repeated Injection of MAb

One patient, who had no anti-mouse globulin antibodies after injection with 366 mg MAb, was reinjected with 150 mg of the same MAb 350 days after the first injection. Sera were obtained 7 and 10 days later that showed the presence of human anti-mouse immunoglobulin antibody, although there were no symptoms of immediate or delayed allergic reactions. A patient who received five injections of 200, 60, 30, 30, and 10 mg of antibody spaced over 11 days has been described previously (1). He did have an anaphylactic type of reaction at the time of injections 11 days following the initial exposure.

DISCUSSION

This report describes the effect of immunotherapy with mouse MAb in a human solid tumor system. The 1083-17-1A MAb chosen for this trial binds to all human CRC maintained in tissue culture (9), and is tumoricidal for all CRC that have been implanted in nude mice (2,3). The results of imaging of CRC in humans with this radiolabeled MAb indicate that the label can be detected in the tumor and in no other tissue on gamma scintigraphy (4–6). Up to 1 g of this purified MAb can be safely given to patients by intravenous infusion without any side effects. The mouse IgG is detectable in the patient's circulation from 2 to 50 days, depending on the dose administered. Confirming the results of imaging, the MAb was de-

tected on cells of metastatic tumor biopsies within the first few days after injection of MAb. When the dose of MAb was ≤200 mg, all patients who were not anergic developed anti-mouse antibodies which were present in the circulation for considerable lengths of time. When the dose of MAb given exceeded 366 mg, eight of nine patients who were otherwise capable of mounting an immune response did not develop anti-mouse antibodies. The one who did had been previously skin-tested with $F(ab')_2$ fragments of the 17-1A MAb for an imaging study. If tolerance was induced by administering large doses of MAb, it could be broken easily by a second administration of an immunogenic dose of MAb. Again, in this case, no untoward reaction was observed. It is quite possible that, to maintain tolerance to mouse immunoglobulin, it may be necessary to continue administration of MAb to a patient at frequent intervals, or large subsequent doses may be necessary to prevent tolerance being broken.

Eleven patients treated in the initial phases of the study had widespread metastases which compromised hepatic or renal function; nine of those also had an abdominal operative procedure. In this clinical setting, we were limited to observations indicating the absence of acute toxicity of intravenously administered MAb and the absence of aggravation of these abnormalities after antibody treatment.

Nine patients whose metastases had not caused hepatic or renal dysfunction are alive 10–35 months after treatment. Three of the nine patients who received 125–611 mg MAb have no evidence of residual disease (nos. 7, 14, and 20 in Table 2). Three of the nine patients failed prior chemotherapy. Although radiation therapy in one patient (no. 9) and chemotherapy in another (no. 13), after MAb treatment, preclude an exact assessment of the role of immunotherapy in these outcomes, one patient (no. 7), who failed previous chemotherapy and received no other treatment following immunotherapy, has no evidence of disease 22 months after MAb treatment.

Although it is difficult to extrapolate results obtained with human tumor xenografts in mice (2,3) to those obtained in humans with gastrointestinal cancer, it is possible that the destruction of tumors in humans occurs because of the tumoricidal activity of the patient's own effector cells mediated by the MAb. In mice the effector cells have been identified as macrophages (3,10); human macrophages isolated from tumor tissue express receptors for the Fc terminal of mouse MAb of IgG_{2a} isotype only (11), and are tumoricidal in the presence of mouse IgG_{2a} MAb. That this is the only mechanism of destruction of malignant cells in humans after treatment with MAb is not clear at present. One cannot rule out the possibility that a human subject may develop his or her own defense mechanism, perhaps through an anti-idiotypic response following exposure to MAb (12).

Taking all this into consideration, we believe that immunotherapy with an MAb that has been proven to specifically destroy human CRC xenotransplants in mice (2,3) may inhibit gastrointestinal cancer in patients with metastatic disease. These results warrant use of this MAb (1) in more extensive clinical trials with patients who have limited tumor burdens.

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Phase II Clinical Trial of a Murine Monoclonal Antibody Cytotoxic for Gastrointestinal Adenocarcinoma[1]

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ABSTRACT

A murine monoclonal antibody (MAb) which binds to human metastatic gastrointestinal adenocarcinomas can be administered safely and has tumor effects in some patients. Its therapeutic effect was assessed in 20 patients with measurable advanced colorectal carcinoma that was refractory to prior surgical resection, chemotherapy, and/or radiotherapy. All patients had agreed to receive no other therapy at the time of MAb administration and follow-up evaluation. In one patient, tumor at all known sites responded after a single i.v. injection of antibody. One other patient had a marked reduction in a hepatic metastasis where binding of ^{131}I-labeled F(ab')$_2$ MAb fragments was demonstrated but not in his abdominal wall metastases where no MAb binding could be demonstrated. In a third patient, stabilization persisting for 12 mo of an aggressively growing tumor was observed. The antibody was well tolerated in all patients, although 10 patients mounted an anti-murine immunoglobulin antibody response.

INTRODUCTION

Immunotherapy of gastrointestinal adenocarcinoma with murine MAb[3] is a new approach to the management of a difficult clinical problem. Previous *in vitro* and animal *in vivo* studies have identified a murine IgG2a MAb 1083-17-1A (17-1A) that has strong cellular effector-mediated cytotoxicity and gastrointestinal tumor inhibitory properties (1-3). Preliminary clinical trials in patients to assess toxicity and binding specificity of this MAb indicated the safety of a single systemic exposure to this murine immunoglobulin, the human response to mouse immunoglobulin as antigen, and the possibility of allergic reactions to multiple antibody infusions (4, 5). The studies also indicated tumor responses in some patients (4, 5). In those patients, biopsy-proven tumor regression was temporally associated with the administration of antibody and 2 of the 20 patients have remained tumor-free for more than 2 yr. Evaluation of antibody specificity in that trial could be done only after biopsy obtained during operative management of metastatic disease and some indicator lesions were thereby altered. Furthermore some of the patients had either stopped chemotherapy just prior to or after entering the immunotherapy trial and one patient received radiation therapy 6 wk after antibody administration, despite the fact that his circulating tumor antigen (carcinoembryonic antigen) levels had already returned to normal.

The present clinical study was conducted to evaluate the effects of MAb 17-1A in a patient population with large tumor burden but without potentially confounding factors such as simultaneous additional forms of therapy. Findings in this trial support a role for MAbs as tumor modulators and suggest the need for modified strategies in antibody administration.

MATERIALS AND METHODS

Patients. Patients with advanced colon and rectal carcinomas were included in this study if they had a 3-mo expected survival, a lesion proven refractory to conventional therapies, a measurable lesion on chest X-ray, liver scan, or computed tomographic evaluations, no other therapy for at least 1 mo prior to mouse MAb administration, no hepatic dysfunction as reflected by bilirubin or serum glutamic oxaloacetic transaminase levels less than twice normal levels, no renal dysfunction, and no other cytotoxic therapy during the 3-mo evaluation phase of the study. A total of 20 patients were included in this study between January and September 1983 with informed consent from each patient. If evidence of disease progression was observed at any time after the start of the evaluation, the patient was treated by the appropriate conventional modalities as determined by the referring physician. The measurable lesions were studied at 1 and 3 mo after antibody infusion by the same technique used for the initial evaluation. Blood studies were done to assess potential hematological, renal, or hepatic toxicity, and to detect immune responses stimulated by murine immunoglobulin.

Antibody. The IgG2a murine monoclonal antibody 17-1A was derived from mice immunized with SW1083 human colon carcinoma cells (6, 7). The antibody was purified from hybridoma cell culture supernatants or from ascites of hybridoma tumor-bearing mice by Protein A-Sepharose column chromatography under sterile conditions. The effluent of the column was tested for Gram-negative endotoxins by the *Limulus* amoebocyte lysate assay. Purity of the immunoglobulin was confirmed electrophoretically. The concentration of IgG was estimated by absorbance at 280 nm. The purified antibody was tested in radioimmunoassay for binding specificities (6, 7). Sterility of the antibody preparation was monitored by incubating a sample for 2 wk in thioglycolate broth and nutrient broth (Difco, Detroit, MI).

Prior to administration, the antibody preparation was centrifuged for 60 min at 100,000 × *g* to remove all immunoglobulin aggregates.

Administration of Antibody. Twenty patients received a single 200- to 850-mg i.v. infusion of MAb 17-1A during the evaluation period (Table 1). In two patients, the infusions had to be discontinued prior to the completion because of urticaria. The antibody was diluted in 250 ml of normal saline and administered over at least 1 h in a Special Care Unit where trained personnel were present to manage potential toxic or allergic reactions. Patients were monitored for changes in pulse, blood pressure, and respiratory function during and after the infusion. Physical examinations were made after completion of the infusion and 1 day later to identify adverse reactions.

Human antimouse antibody response was assessed before and 1 wk after MAb infusion (4, 5). No patient with preexisting antibody against murine globulin was identified. Sera reactive after MAb administration

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[3] The abbreviations used are: MAb, monoclonal antibody; CAT, computerized axial tomography.

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Table 1
Phase II clinical trial with MAb

Patient	Sex	Age	Site of primary	Site of metastasis	MAb dose (mg)	Carcinoembryonic antigen at time of antibody administration	Anti-mouse antibody	Anti-idiotype antibody	Status
21	M	60	Colon	Liver	765	2540	–	–	Dead
22	F	40	Colon	Lung	255	246	–	–	Dead
23	M	65	Colon	Liver	850	51	+	+	Dead[a]
24	M	58	Colon	Liver	800	260	+	–	Dead
25	M	41	Rectum	Lung	112	293	+	+	Dead
28	M	51	Colon	Retroperitoneum	200	76	+	+	Dead
27	F	49	Rectum	Retroperitoneum	770	9.4	+	+	Dead
28	M	65	Rectum	Pelvis	192	81	–	–	Dead
29	F	48	Colon	Lung	207	<2.5	+	+	Dead
30	F	65	Colon	Liver	215	96	–	–	Dead
31	M	55	Colon	Liver	220	1080	–	–	Dead
32	F	36	Colon	Pelvis	220	<2.5	–	–	Alive with disease[b]
34	F	70	Rectum	Pelvis	220	<2.5	–	–	Alive with disease[b]
35	F	44	Colon	Pelvis	220	<2.5	+	+	Dead
36	M	62	Colon	Liver	220	48.2	–	–	Dead
37	M	47	Colon	Pelvis	207	<2.5	+	–	Dead
39	M	53	Colon	Lung	200	12.6	–	–	Alive with disease
41	M	57	Colon	Lung	200	<2.5	+	+	Dead
42	M	60	Colon	Lung	200	4.4	+	+	Alive with disease
43	F	65	Colon	Retroperitoneum	200	33.8	–	–	Alive with disease

[a] Response in liver metastasis but not in abdominal wall metastasis.
[b] Responders.

were further assayed for the appearance of antiidiotypic antibody weekly for 4 wk and then monthly for 3 mo (8). Patients were examined for signs and symptoms of serum sickness for 1 mo after the infusion.

RESULTS

Most patients tolerated the infusion well. Two patients developed urticaria, one of whom was treated with antihistamine. One patient developed nausea and another developed diarrhea in the immediate postinfusion period but these symptoms spontaneously resolved. There were no vasomotor changes, bronchospastic complaints, nor any delayed symptoms that could be associated with serum sickness. No hematological, hepatic, or renal changes were noted. Ten of 20 evaluable patients developed human anti-murine antibody. Eight of 10 patients showed anti-idiotype antibody responses, two patients did not raise such an immune response, and the response in the other patient has not been determined.

Three of 20 patients had tumor effects which were documented at 1 and 3 mo after administration of MAb and persisted for more than 12 mo. One of these patients (Table 1, patient 32) responded partially with resolution of a pelvic recurrence as detected by CAT at 1 and 3 mo after antibody administration (Figs. 1 and 2). At 18 mo after antibody treatment, no recurrence of that mass has been detected, although that patient's carcinoembryonic antigen levels which were normal for 12 mo are now increasing. The site of her metastatic disease is a small pulmonary nodule. The second responding patient (Table 1, patient 34) was judged to have a stabilization of a pelvic recurrence which had been growing rapidly prior to antibody infusion. Disease in this patient has remained stabilized for 12 mo since treatment, as indicated by an unchanged CAT scan and the patient has improved symptomatically.

Of the 18 remaining patients, one (patient 23) had previously been included in a radioimaging trial using iodinated $F(ab')_2$ fragment of MAb 17-1A. In that trial, antibody had localized in his large hepatic tumor but not in his abdominal wall metastasis. In the present immunotherapy trial, the hepatic metastases of this patient showed partial regression and calcification, as documented by CAT scan, while the abdominal wall metastasis continued to grow. Because of the progresion at the latter site, the patient was considered a nonresponder.

DISCUSSION

The murine MAb 17-1A has been an extremely valuable reagent for the study of human gastrointestinal adenocarcinoma (1–12). It binds to almost all colon adenocarcinoma cell lines tested and can be used to radioimage metastatic tumors (6, 7, 11, 12). Radiolocalization techniques can detect disease using monoclonal reagents not identifiable by conventional CAT scan evaluations (12).

The patient population in the present study differed considerably from that in the phase I study (4, 5). Patients in the present study had large tumor burden, a short expected survival time, and were required to cease all other therapies for a full month before entering the immunotherapy trial. Furthermore many patients in the first trial received single or multiple injections of MAb incubated with an autologous leukocyte preparation (4, 5), whereas patients in the present trial received MAb alone and in a single administration. Modification of any one of these parameters might alter the ultimate outcome of MAb therapy for the patient.

The heterogeneity of antigen expression in disseminated metastatic tumor, as demonstrated by the response of a patient's hepatic metastasis but not by the abdominal wall metastasis, is of interest for future approaches to MAb immunotherapy but thus far has been observed in only 2 patients of our total series. Since other investigators (13) have demonstrated that antibody in cooperation with host effector cells can kill tumors of small size, it is impressive that some patients with larger tumor burden had also responded. As MAb 17-1A seems to exert its effect through armed macrophage mediators (3, 14), the minimal response in some of the patients may rest in the unfavorable ratios of these effector cells to billions of proliferating tumor cells. It is also worth stressing that the effector cell function in many

patients was impaired by previous treatments such as chemotherapy and radiation.

Of the group of patients described previously (8) and those included in the present study, seven had tumors that were altered in response to MAb treatment, and in 5 of these patients in the original study, antiidiotypic responses were demonstrated. Only one of the two responders with large tumor burden in the present study had detectable anti-idiotype antibodies.

These results support previous observations that a murine cytotoxic anticolorectal cancer MAb can modulate growth of adenocarcinoma in patients. Evidence continues to hold for variable responses in patients with large tumor burdens. Future studies in patients with small residual tumor burden are needed. Strategies that allow multiple exposures to MAbs reacting with different antigens on the cancer cells should allow more efficient treatment and overcome the problem of tumor cell heterogeneity.

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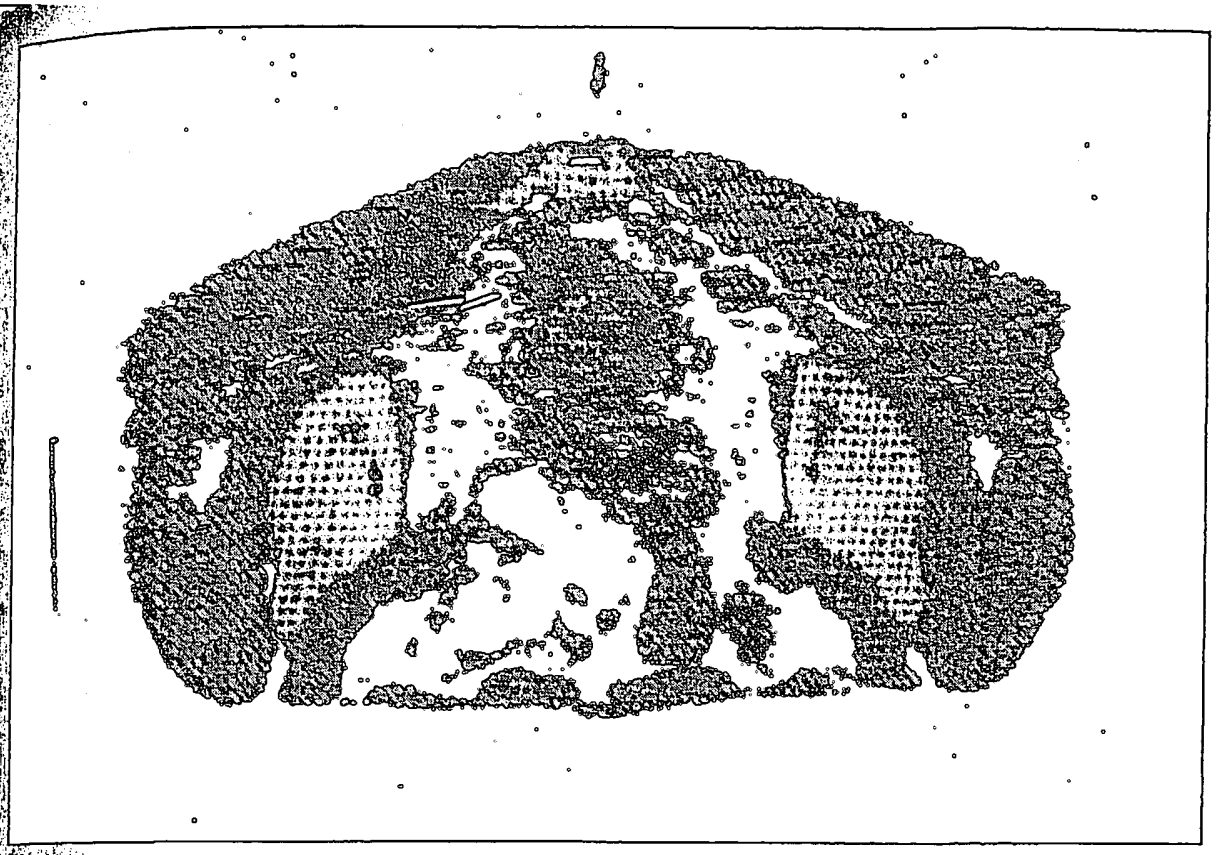

Fig. 1. CAT scan of pelvis in patient 32 taken before MAb administration delineates tumor area (*arrow*).

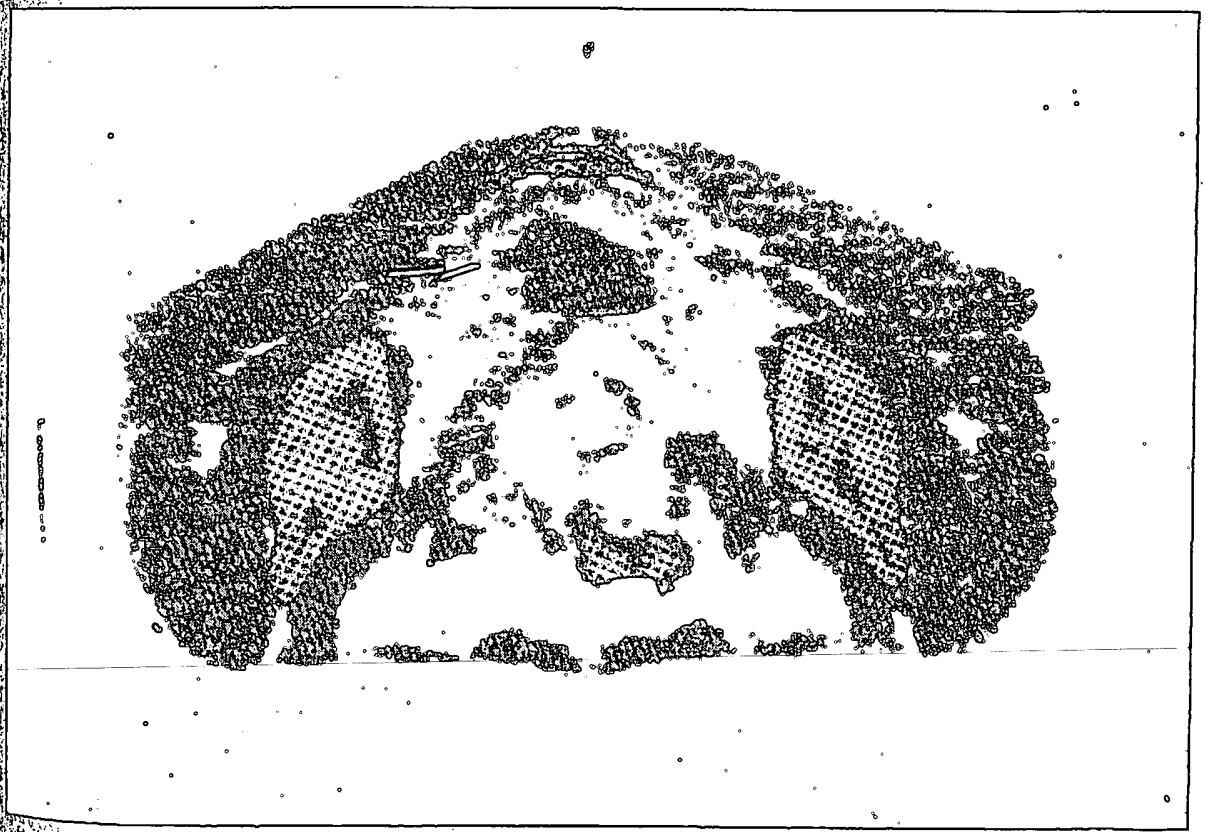

Fig. 2. CAT scan of pelvis at similar levels of the lesion (see Fig. 1) taken 4 mo after administration of 250 mg of MAb 17-1A. Note the marked reduction in the tumor area (*arrow*).

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Immune Response to Tumor Antigens in a Patient with Colorectal Cancer after Immunization with Anti-idiotype Antibody

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An active vaccination protocol was performed on one patient with colon carcinoma as a pilot to a prospective randomized double-blind clinical trial with the vaccine SDZ SCV 106. This vaccine is an anti-idiotype goat antibody to the monoclonal antibody 17-1A, which is directed against the tumor antigen 17-1A. To study the effect of the therapy on the immune reactivity, several tests were performed to detect anti-tumor antibodies in the serum as well as in cluates of metastatic tissue. Furthermore metastases removed from the lung were examined by immunohistochemistry. The results suggest that the humoral and cellular immune reactivity against the tumor are enhanced. © 1992 Academic Press, Inc.

INTRODUCTION

Many approaches to enhance immunological tumor rejection have been used or are still under investigation. Passive serotherapy with monoclonal antibodies could eradicate clinically evident established tumor in some patients with gastrointestinal carcinoma (1–3). The main disadvantage of a treatment with animal monoclonal antibodies is the development of antibodies to the species-specific parts of the antibody, which can neutralize therapeutic effects and cause adverse events, as reported by many authors. A novel approach is the development of human antibodies to tumor antigens. Besides doing this *in vitro*, it is also possible to elicit an immune response *in vivo*, making use of anti-idiotype antibodies bearing the internal image of tumor antigens. Vaccination by this anti-idiotype principle has been suggested to be suitable for situations in which the tumor antigen itself cannot be purified or safely administered. Although animal experiments demonstrate the validity of this approach (4), no randomized clinical trials on the efficacy of this therapy have been reported so far.

An anti-idiotype vaccine, SCV 106 (5), was developed in goats against a monoclonal antibody 17-1A from the Wistar Institute, which is directed against the antigen 17-1A, present on colon carcinoma cells (6). This vaccine can be expected to bear the internal image of the tumor antigen and might therefore induce an immune response against the tumor. A Phase I study has already been published (6). As a pilot to a prospective randomized double-blind clinical trial, one patient was treated with SCV 106. Immune reactions in this patient were studied.

PATIENTS, MATERIALS, AND METHODS

Patients

The pilot patient was a 39-year-old man who was hemicolectomized due to colon carcinoma (Dukes C). At the time of entry into the study (2 years after operation) he had two lung metastases. The immunization treatment with goat anti-id 17-1A consisted of sc injections, 4 mg each, in Weeks 0, 1, 2, 4, 10, 16, 22, and 28. The therapy was given with informed consent. By the end of therapy, no new metastases were found. The two lung metastases, which by CT scan appeared to have slightly increased in size to 3 and 1 cm in diameter, were then surgically removed. During the course of the study the presence of specific B-cells and antibody titers against anti-id 17-1A were investigated. The removed metastatic tissue was submitted to immunohistochemical and biochemical investigation.

Control sera were taken from a placebo patient taken from the double-blind clinical trial, which is currently in evaluation. This control patient was a 42-year-old woman operated on for colon carcinoma 1½ years before entry in the study. She had two liver metastases. Controls in the double-blind study were treated with the placebo vaccine, normal goat antibody. The treatment scheme was identical to that described above for the anti-id treatment.

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Control B lymphocytes were taken from an untreated colon carcinoma patient with comparable metastatic disease.

Preparation and Purification of the Anti-idiotypic Antibody, SCV 106

Goats were injected subcutaneously at multiple sites with purified antibody 17-1A emulsified in complete Freund's adjuvant. Booster injections with antibody in incomplete Freund's adjuvant were applied on Days 8 and 29. Serum was collected over a period of 2 months and IgG was isolated from goat immune serum by ion-exchange chromatography on DEAE Trisacryl M BF). Isolation of anti-idiotypic antibodies was done by immuno-affinity chromatography. Immunosorbents (mouse IgG2a-Sepharose, 17-1A-Sepharose, and polyclonal mouse IgG-Sepharose) were prepared by coupling purified antibodies to CNBr-activated Sepharose 4B (Pharmacia). Goat IgG was sequentially adsorbed on mouse IgG2a-Sepharose and 17-1A-Sepharose to remove anti-isotypic and anti-idiotypic antibodies, respectively. The fraction containing anti-idiotypic antibodies was passed over the polyclonal mouse IgG-Sepharose to remove trace amounts of anti-isotypic antibodies. Purity of isolated anti-idiotypic antibodies with reference to the anti-isotypic cross-reaction, tested by ELISA, was better than 99%.

Detection of Human Anti-Goat Antibodies

Total humoral immune response was evaluated by measuring antibody titers against the immunizing agent by an ELISA system (7). Microtiter plates (Immunoplate II, Nunc) were coated for 1 hr at room temperature with the immunizing agent (goat anti-id 17-1A) at 2 μg/ml in carbonate buffer (0.05 *M*, pH 9.6), blocked with a 5% fetal calf serum (FCS) solution in PBS; after extensive washing, serial dilutions of serum samples were incubated for 1 hr at room temperature. The plates were then washed and an excess of peroxidase-labeled sheep anti-human Ig (Amersham) was added and incubated for 1 hr at room temperature. After washing, ABTS (Amersham) was added to react for 15 min. Absorbance was read at 405 nm with a 620-nm reference wavelength. Results were expressed as reciprocal serum dilutions at the 20% level of the maximum absorbance.

Detection of Specific Immune Response (Anti-id Titer)

Human antibodies that inhibit binding of the vaccine (anti-id 17-1A) to moab 17-1A were measured to evaluate the specific (anti-idiotypic) immune response (6). Serum samples were preincubated with normal goat IgG at 300 μg/ml overnight at room temperature. Serial dilutions of all samples were made and incubated with biotinylated goat anti-id 17-1A at 1 μg/ml for 30 min at 37°C. Microtiter plates (Immunoplates II, Nunc) were coated overnight at 4°C with moab 17-1A at 0.3 μg/ml in carbonate buffer (0.05 *M*, pH 9.6) and blocked with a 5% fetal calf serum (FCS) solution. After extensive washing, preincubated serum samples were transferred to the plate and incubated for 30 min at 37°C. The plates were then washed and an excess of peroxidase-conjugated avidin D (Vector Laboratories, California) was added for a 30-min incubation at 37°C. After washing, ABTS (Amersham, UK) was added and reacted for 15 min. Absorbance was read at 405 nm with a 620-nm reference wavelength. Results were expressed in reciprocal serum dilutions at the 50% level of the maximum absorbance.

Detection of Tumor Cell Binding Antibodies (MHA)

Sheep red blood cells (SRBC) were coated with goat anti-human Ig antibodies (Amersham) using a 0.1% $CrCl_3$ and 0.9% NaCl solution.

Tumor cells (SW 1116) in MEM containing 10% FCS were plated (70,000 cells per well) in a microtiter plate (Nunc) and incubated for 24 hr at 37°C. After washing with PBS, serial dilutions of serum were added and incubated for 1 hr at room temperature. The plates were then washed and the coated SRBCs were added for a 1-hr incubation at room temperature. After washing, about 100 cells of each well were scored microscopically (7). Cells were scored positive if at least two erythrocytes were attached.

Detection of Antibodies Binding to Purified 73-3 Tumor Antigen

Tumor antigen binding human antibodies, produced by EBV immortalized B cells (8), were detected by an ELISA system. Microtiter plates were coated overnight at 4°C with 73-3 tumor antigen (7) using Biobeads (SM4, Bio-Rad, Austria) and blocked with a 3% bovine serum albumin (BSA) solution. After extensive washing, immortalized B-cell supernatants were incubated

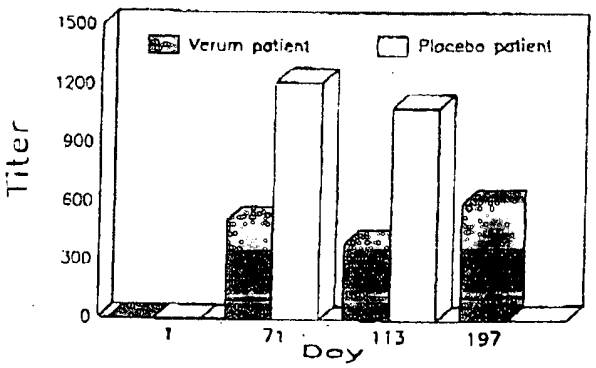

FIG. 1. Total humoral immune response against the immunizing agent. SDZ SCV 106 was used as verum vaccine and normal goat antibody as placebo vaccine.

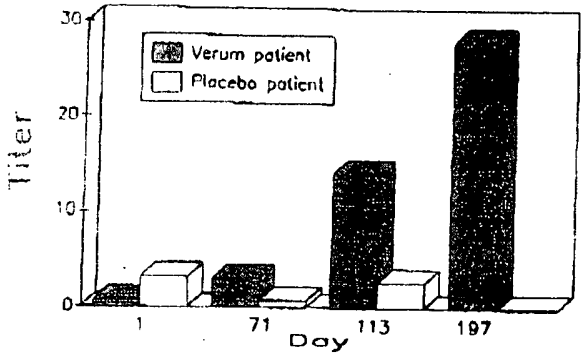

FIG. 2. Humoral immune response in a competitive ELISA system using the vaccine 17-1A as antigen. Antibodies inhibiting the binding of moab 17-1A to the vaccine are shown.

% Positive Wells		
	Verum Patient Week 31	Untreated Patient (Colon Tumor)
Weak	18	1
Medium	4	0.7
Strong	3	0.5

FIG. 4. EBV-immortalized patient's B cells produce antibodies against the tumor antigen, compared to B cells taken from an untreated colon carcinoma patient.

for 1 hr at 37°C. The plates were then washed and an excess of peroxidase-labeled sheep anti-human Ig (Amersham) was added for a 1-hr incubation at 37°C. After washing, ABTS (Amersham) was added and reacted for 1 hr at room temperature. Absorbance was read at 405 nm with a 620-nm reference wavelength.

Immunohistochemistry

Five-micrometer thin cryostat sections of metastatic tissue were prepared, fixed in acetone, and then preincubated with hydrogen peroxide to destroy endogenous peroxidase and with normal goat serum to prevent unspecific binding of antibodies. Subsequently the slides were incubated with primary mouse monoclonal antibodies anti-CD3 (T cells), anti-CD4 (T-helper cells), anti-CD8 (T-suppressor/cytotoxic cells), anti-CD14 (monocytes/macrophages), and CD19 (natural killer cells) (Becton Dickinson, California), with the 73-3 antibody reacting with the same antigen as anti-17-1A produced by Sandoz, Vienna, Austria) and with the anti-HLA-DR, which was a generous gift of Dr. O. Majdic (University of Vienna, Austria). Normal mouse IgG was used as a negative control. Furthermore, the polyclonal rabbit anti-human C3C antiserum (Dako, Denmark) was used, with unspecific rabbit serum as a negative control. Following repeated washing, goat anti-mouse antibody (Nordic, The Netherlands) or swine anti-rabbit FITC (Dako, Denmark) was added to the slides. The slides with mouse monoclonals as first antibody were treated with mouse peroxidase–anti-peroxidase (PAP) complex (Dako, Denmark) and peroxidase demonstrated by diaminobenzidine (Fluka, Switzerland). These sections were counterstained with hematoxilin, dehydrated, and coverslipped with Permount. The slides stained with the FITC conjugate only were washed and mounted.

Elution of Antibodies from Metastatic Tissue

Parts of the metastatic tissue were used for elution of eventually bound anti-tumor antibodies. The tissue was mechanically dissociated and extensively washed

Serum Dilution	Day							
	1		71		113		197	
	placebo	verum	placebo	verum	placebo	verum	placebo	verum
1	-	-	-	***	-	***	-	***
3	-	-	-	***	-	***	-	***
6	-	-	-	**	-	***	-	***
12	-	-	-	*	-	**	-	***
24	-	-	-	-	-	*	-	**

FIG. 3. Tumor cell binding antibodies were found in the serum of the patient treated with the verum vaccine and not in the serum of the placebo patient. 10–20% positive cells, *; 20–50%, **; >50%, ***.

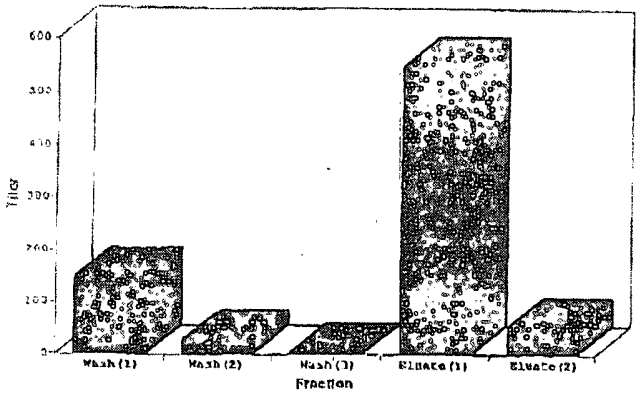

FIG. 5. Presence of antibodies binding to the immunizing agent (anti-id 17-1A) in ELISA titer units. Low titers of antibodies could be detected after the first washing. The eluate after 1 hr incubation contained the highest titer of antibodies. Only minute amounts of antibodies were eluted thereafter.

with PBS by gently shaking it at room temperature. Buffer was changed several times until no human antibodies could be detected in the supernatant. Acidic buffer treatment (0.2 *M* Glycil/HCl, pH 2.8) for 1 hr at room temperature was then performed for elution of antibodies tightly bound to the tissue (eluate 1); thereafter the tissue was incubated with the same buffer for another 30 min (eluate 2). The supernatants were investigated for the presence of antibodies binding to the immunizing agent (anti-id 17-1A) in ELISA.

RESULTS

Figure 1 shows the total immune response against the immunizing agent which remained constant during the whole immunization period in both cases.

The patient developed a specific immune response in the competitive ELISA system, in terms of an anti-anti-id titer, which rose quite slowly during the treatment period. No such specific titer could be detected in serum samples from the placebo patient, as demonstrated in Fig. 2.

Tumor cell binding human antibodies could be found in serum samples of the patient using the MHA technique, in contrast to the control samples. These results are shown in Fig. 3.

Using EBV immortalized B cells of the patient, an increasing number of clones was observed during therapy producing antibodies specific for the 17-1A tumor antigen. A comparison of clones from the patient 31 weeks after initial immunization with those from an untreated patient is shown in Fig. 4.

The antibody elution method performed on the metastases showed antibodies binding to the immunizing agent (anti-id 17-1A) in ELISA (Fig. 5).

The metastatic tissue removed from the lung consisted of various sized glands which were lined with several irregular layers of colonic epithelial cells and which contained numerous mitotic figures. Immunohistochemical reaction utilizing 78-3 antibody showed that the epithelial component of the metastasis did express the examined tumor antigen, since the moab 78-3

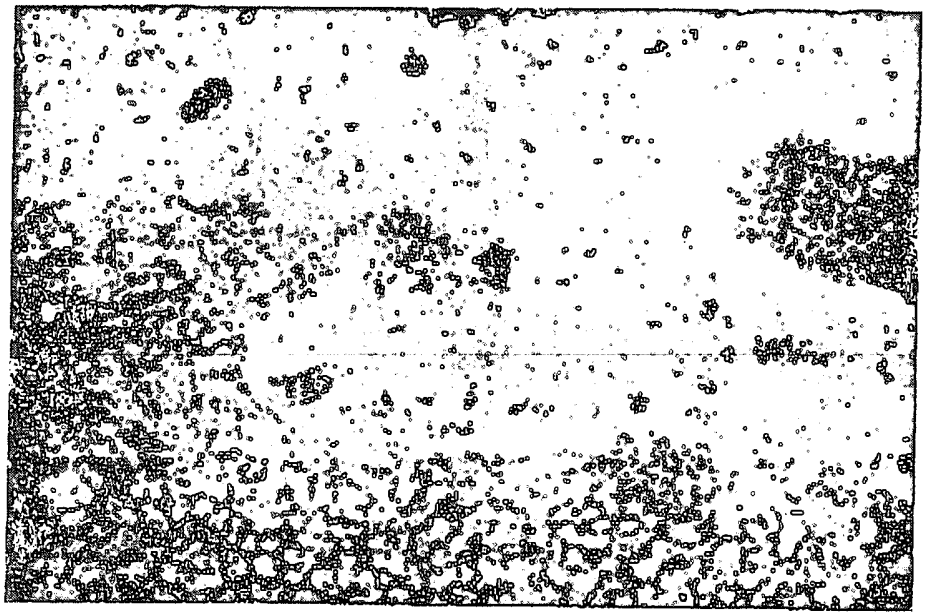

FIG. 6. Immune-peroxidase reaction with the moab 73-3 performed on metastatic tissue, removed from the lung. The metastatic cells strongly express the tumor antigen, to which the anti-id, used for the described vaccination, was an internal image (see arrow). A group of lymphocytes (see double arrows) infiltrates the stroma surrounding metastatic epithelial cells. Magnification ×400.

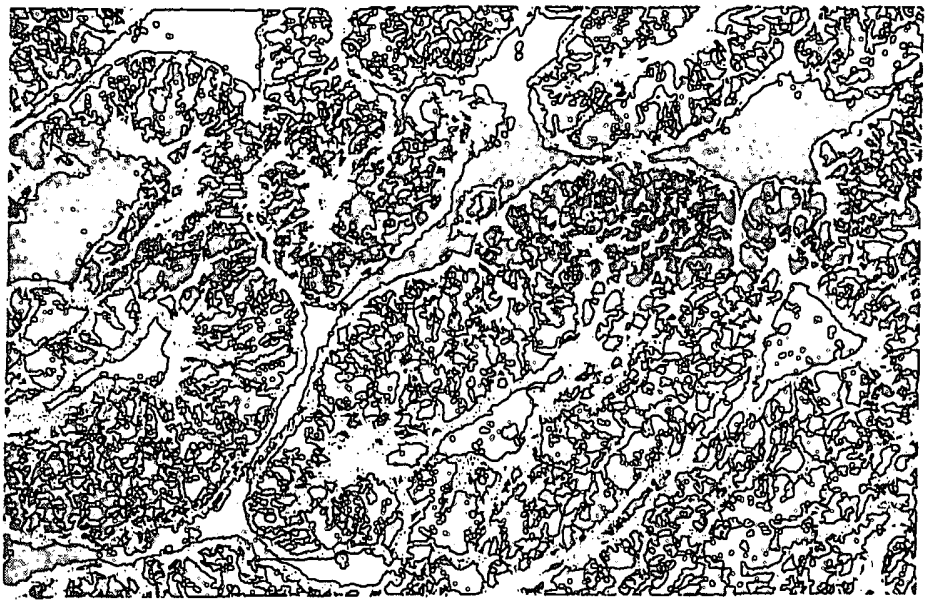

FIG. 7. Immunofluorescence: The same tissue as that shown in Fig. 6 reacted with polyclonal anti-complement antibody. Tumor cells are positive. Magnification ×400.

showed strong positivity. This moab detects an antigen identical to the 17-1A antigen (Fig. 6). About one-third of the metastatic tissue examined consisted of necrotic foci, some of which contained small mononuclear cells with pycnotic nuclei. Several parts of the metastases showed a varying degree of desmoplastic reaction. Some stromal territories contained groups of infiltrating lymphocytes. Alveolar macrophages with balloon-like cytoplasm filled the compressed alveolar lumina in the areas surrounding the metastasis. Metastatic epithelial cells showed a diffuse staining pattern for the complement component C3c (Fig. 7). They also were strongly HLA-DR positive (Fig. 8). The bed of the metastasis was composed of a narrow layer of partly ne-

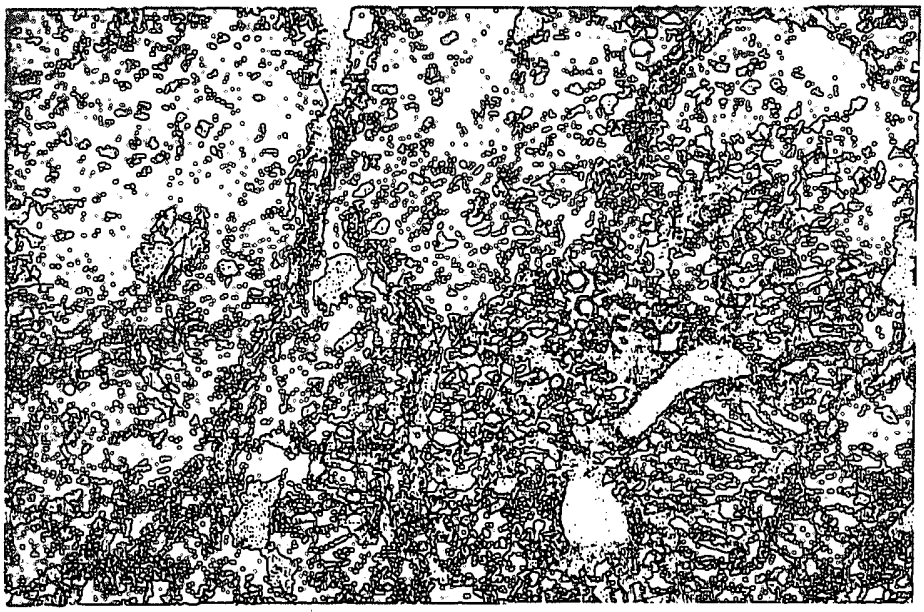

FIG. 8. Immuno-peroxidase reaction on the same tissue as that in Fig. 6 with a monoclonal antibody directed against HLA-DR. The metastatic epithelial cells showed a strong focal reactivity (see arrows). Magnification ×400.

crotic, compressed lung tissue and an extended conglomerate of densely packed lymphocytes (Fig. 8). Immunostaining showed that most of these lymphocytes were T cells ($CD3^+$), predominantly T-helper cells ($CD4^+$, Fig. 9); only very few were T-suppressor/cytotoxic cells ($CD8^+$, Fig. 10). Lymphocytes localized within the tumor stroma were mostly $CD3^+$ and showed CD4 or CD8 positivity in an equal distribution. We failed to classify the small mononuclear cells with pycnotic nuclei present in the necrotic tumor tissue as they were $CD3^-$, $CD14^-$, and $CD19^-$.

DISCUSSION

The presented serological findings indicate that a specific humoral immune response against tumor is induced in this pilot case of an active immunization treatment with the anti-idiotype vaccine SDZ SCV 106. Although the control patient treated with a vaccine consisting of unspecific normal goat immunoglobulins showed a stronger overall humoral response, she apparently had no immune response to tumor-specific antigens. Therefore these preliminary data give support to the hypothesis that the humoral tumor defense is enhanceable with the immunization protocol used.

Many studies address the question of whether circulating anti-tumor antibodies can penetrate tumor tissue. In this case they did, as antibodies reactive in the anti-tumor antigen ELISA could be eluted from the metastatic tissue. Furthermore, there is good evidence that they were functional in complement activation, as complement binding could be detected using immunohistochemistry. The presence of a massive infiltrate of T-helper cells in the metastasis bed indicates that the metastatic tissue removed from the lung is recognized by the cellular immune system. These CD4-positive T cells may help B cells in secreting antibodies to the vaccine and to the tumor, whereas the cytotoxic T cells might cause necrosis of the tumor (9). The strong focal appearance of HLA-DR on tumor cells could be due to local cytokine release. HLA-DR positive tumors are known to contain more tumor-infiltrating lymphocytes (10). The results from the double-blind clinical trial will give information as to whether this enhanced immunological recognition of the tumor has any impact on the courses of disease or on survival time.

FIG. 9. Immunostaining on lymphocytes infiltrating the partly necrotic lung tissue, using anti-CD4. T-helper cells predominate. Magnification ×350.

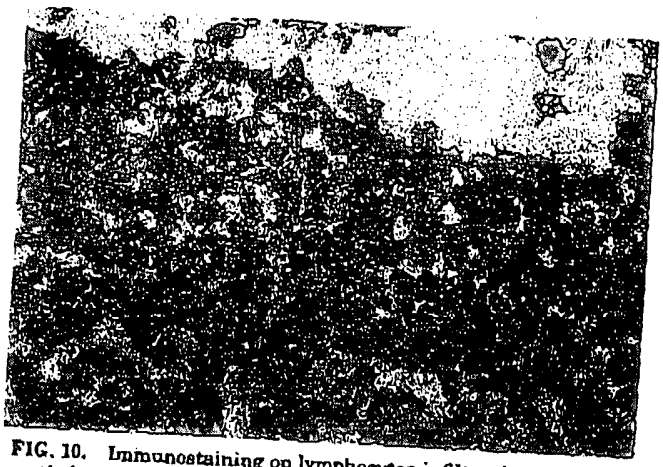

FIG. 10. Immunostaining on lymphocytes infiltrating the partly necrotic lung tissue, using anti-CD8. T-suppressor cells were only sparse. Magnification ×350.

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Antibody engineering

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The development of recombinant techniques for the rapid cloning, expression, and characterization of cDNAs encoding antibody (Ab) subunits has revolutionized the field of antibody engineering. By fusion to heterologous protein domains, chain shuffling, and inclusion of self-assembly motifs, novel molecules such as bispecific Abs can now be generated which possess the subset of functional properties designed to fit the intended application. Rapid technological developments in phage display of peptides and proteins have led to a plethora of applications directed towards immunology and antibody engineering. Many of the problems associated with the therapeutic use of Abs are being addressed by the application of these new techniques.

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Abbreviations

Ab	antibody
CDR	complementarity determining region
EPO	erythropoietin
ES	embryonic stem
IL	interleukin
m	monoclonal
PCR	polymerase chain reaction
SC	single chain
V	variable
YAC	yeast artificial chromosome

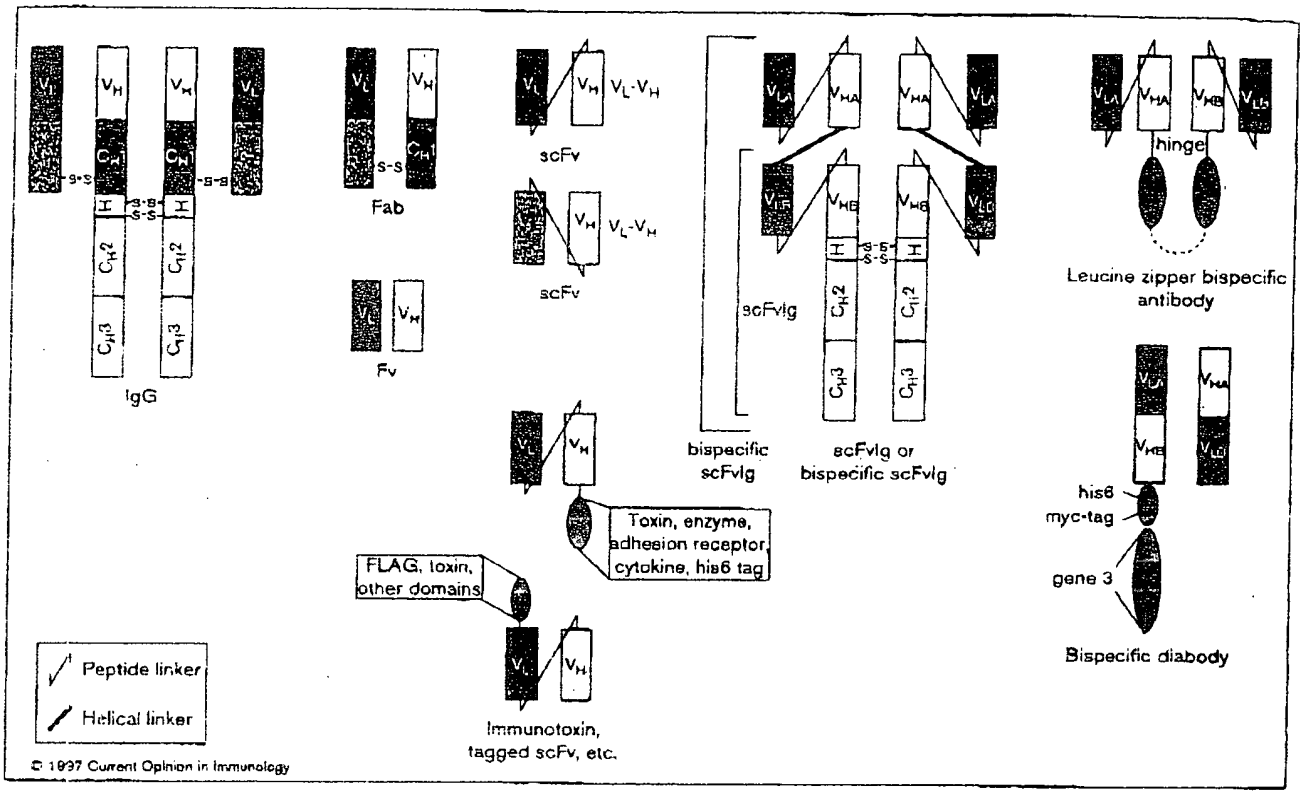
Introduction

Antibody (Ab) molecules and their derivatives have tremendous potential for use in a variety of research, diagnostic, and therapeutic applications. Recent progress in the manipulation of Ab subunits using molecular techniques, plus the ability to reproducibly select an Ab or potential binding target, now allows the generation of useful binding subunits that function as expected, while avoiding many of the problems that have often been associated with these molecules. The domain structure of Abs permits their division into functional subunits, or modules, which can be mixed and matched to create novel molecules with a specific subset of functional properties. The constant regions of the Ab molecule contribute effector properties including Fc receptor binding, serum half-life, and complement fixation. This review, however, concentrates on engineering the antigen-binding site, which is determined by the variable (V) regions of the heavy (V_H) and light (V_L) chains of the Ab molecule. The antigen-binding site is formed by six hypervariable loops known as complementarity determining regions (CDRs), three derived from the V_H chain (H1, H2, and H3), and three from the V_L chain (L1, L2, and L3), which extend beyond the scaffold formed by the V_H and V_L framework regions. The CDR loops form highly exposed domains that interact with antigen, yet available evidence suggests that only a limited number of CDR residues actually participate in direct antigen-binding interactions [1]. The genes encoding V_L and V_H domains can be subcloned and fused to create an engineered gene encoding a single chain (sc)Fv molecule. ScFv fragments form functional derivatives of Abs that maintain the monovalent binding specificity of the original intact Ab molecules. To create a single fusion protein and to stabilize the interaction between the V_L and V_H domain of the scFv molecule, V_L and V_H are separated by a peptide linker of variable length and sequence [2–6], although the most common linker used has been the $(Gly_4Ser)_3$ flexible linker originally used by Huston *et al.* [7]. Figure 1 illustrates the domain structure of Abs and also diagrams some of the novel molecules created from Ab subunits. This review focuses on recent developments in the field of Ab engineering, particularly in the areas of gene cloning, expression systems, humanization of Abs, bispecific Abs, and small antigen-binding Ab fragments.

Advances in gene cloning techniques

Ab engineering evolved rapidly following the development of sophisticated techniques based on polymerase chain reaction (PCR) cloning and amplification of genes and gene libraries [8]. The generation of a substantial database of Ab V region sequences has not only permitted the design of V region specific oligonucleotides for further PCR applications, but has also facilitated advances in the design, computer modeling, and humanization of Ab derivatives. Generally, cDNAs from hybridomas or other cells producing a defined Ab are used to clone the sequences encoding the V domains of Abs, and methods for the rapid and reliable cloning of hybridoma V regions have been reported in the past two years [9•,10]. Alternative methods have utilized various phage display vectors and gene fusion techniques, along with PCR amplification of genes for Ab subunits, to generate Ab repertoires *in vitro* [11,12•]. PCR amplification of V regions from hybridomas [13], from preimmunized donors [12•,14], from naive donors [12•,15] or from human germline sequences with semisynthetic inserts of randomized CDR3 regions [16••] have been used to generate single scFv–phage fusions or diverse combinatorial Ab libraries.

Figure 1



Structure of Ab molecules and their derivatives. The schematic structure of a native Ab molecule (IgG) and derivatives composed of Ab subunits are diagrammed. For each molecule, the amino terminus is oriented toward the top of the figure, while the carboxyl terminus is oriented toward the bottom of the figure. Drawings are not to scale, and the peptide or helical linkers are usually fairly short domains of less than 30 amino acids in length. Solid lines indicate peptide domains, while dotted lines between two domains indicate that they associate with one another to form heterodimers. C_H, constant heavy chain domain; C_L, constant light chain domain; H, hinge; scFv, single chain variable fragment; s-s, disulfide bond; V_L, variable light chain domain; V_H, variable heavy chain domain. His6 refers to a short peptide sequence tag which adds a purification domain to the Ab fragments. Similarly, myc, FLAG and HA tags are all short immunogenic peptides recognized by antibodies and provide an identification and possible purification domain for the molecules to which they are attached. Gene 3 protein is a bacteriophage minor coat protein to which other fusion proteins or peptides can be attached for phage display. Depending on the vector and the coat protein used for creating the scFv-phage fusion proteins, monovalent or multivalent display can be achieved. Fos and jun are leucine zipper sequences which interact to drive heterodimerization (fos pairs with jun) of the molecules to which they are attached.

Although the general methods mentioned here are not new, the combined use of these existing methods and the compilation of more complete sequence databases has permitted researchers in the field to streamline techniques so that more rapid and reliable cloning of scFvs can be achieved. The various methods have been developed with particular applications in mind, so that different techniques are utilized for cloning a desired mAb specificity from an existing hybridoma or for isolating new molecules specific for a desired target antigen from naive repertoire libraries. Each approach has its advantages and disadvantages, so that the choice of methods for isolating these molecules often depends on how the antibody binding specifities are to be used and whether an antibody already exists with desirable properties.

A novel method of cloning Ab cDNAs nicknamed SLAM (selected lymphocyte antibody method) increases the power of scFv cloning technology by permiting the selection and isolation of single lymphocytes producing Abs against rare defined target antigens using hemolytic plaque assays with antigen coated erythrocytes [17••]. Single lymphocytes are recovered by micromanipulation, and then reverse transcription (RT)-PCR amplification of V_H and V_L cDNAs is used to clone, express, and reconstitute the monoclonal (m)Abs of each cell. Once the cDNAs for scFv molecules are isolated by one of the above methods, they can be used alone to express bare scFvs, or as the building blocks for gene fusions to create artificial adhesion receptors [18•], immunotoxins [19–22], bispecific Abs [23–35], intracellular agents which disrupt target

antigen function [9•,36•,37–39,40•,41•,42], or bispecific proteins such as cytokine or enzyme fusions [43,44].

Expression systems

Depending on the application and complexity of the recombinant engineered molecules, a particular expression system may be preferable to others. A variety of transient and stable mammalian expression systems for Ab subunits and gene fusions are currently in use [45•]. Transient expression systems involving COS cells have been developed for the rapid and reliable construction, expression, and screening of multiple whole Abs [46–49], Ab derivatives [10,24,35,47], and various heterologous gene products [43,50,51]. Abs and their derivatives can be expressed by inserting cDNA or genomic cassettes under the control of viral or cellular promoter and enhancer elements. Transfection can be performed by several methods involving diethylaminoethyl (DEAE)-dextran [24,49], calcium phosphate [52], electroporation [46], lipofection [45•], or even retroviral transduction [18•].

Expression levels of Ab derivatives from COS cells may vary from less than 0.1 mg ml^{-1} to 10 mg ml^{-1}, depending on the fusion construct, expression vector, and the actual V region sequences [10,45•]. The variability in expression levels is probably a function not just of the construct itself, but also of the host cell used for expression. In particular, if COS cells express antigen which cross-reacts with the scFv being expressed, then the expression level will be lower than normal. We have observed lower expression levels of anti-human CD40 scFv molecules which cross-react with the monkey CD40 expressed on COS cells. In addition, some variability in scFv expression levels has been observed using different cell lines, although several constructs which express poorly in COS also do so in other host cells (M Hayden, A Watson, unpublished data). COS cells are monkey kidney cells transformed by SV40, an oncogenic polyoma virus capable of transforming human cells, and are also an adherent cell line, two less desirable properties for large scale production because these molecules might be used in humans and because suspension cells are more flexible for scaleup and production, requiring no surface matrix for growth and maintenance of the culture. CHO and myeloma cell lines can be used for transient transfection but, in general, adherent cell lines such as COS cells are ideal for rapid transfection protocols and simplify the manipulations involved in the harvest and screening of culture supernatants.

As examples, both dihydrofolate reductase (DHFR)-CHO and myeloma cell types can be transfected, adapted to serum-free conditions, and grown to high densities in suspension culture, and the protein can be expressed at high levels with or without amplification [45•]. Serum free conditions decrease the costs of producing antibody derivatives, and eliminate a possible source of contamination by virus, prions, or other infectious material. Myeloma lines such as SP2/0, YB2/0, NSO, and P3X63.Ag8.653 [45•,53•], and DHFR-CHO lines such as DUKX-B11 and DG44 [54] can be used for stable, high level expression of Ab derivatives. Several methods have been developed for co-amplifying expression of a selectable marker gene and the Ab derivative using glutamine synthetase (GS), adenosine deaminase (ADA), neomycin (Neo or G418), and DHFR [55,56] as marker genes. The selective agents used vary in price and depend on the host cell used for expression. Other improvements in serum-free growth methods, such as the use of fed-batch culture of mammalian cell lines and the careful monitoring of nutrient metabolism, permit alterations in the recipes and schedules for feeding, and have led to increases in the yield of Ab derivatives [57,58]. For bispecific Ab derivatives constructed as single chain gene fusions, these mammalian systems are more likely to generate properly folded, functional gene products with a much higher efficiency than bacterial or phage display type systems [27].

Bacterial expression technologies are constantly undergoing refinements and improvements, yet the scFvs expressed are often improperly assembled, unglycosylated, and misfolded, requiring renaturation and other manipulations to generate functional protein [27,45•,59]. Despite these problems, bacterial expression has a number of advantages over mammalian expression systems, including rapid generation time, ease of cloning, and induction levels that can reach up to 30% of total bacterial protein. Two general strategies have been used for *Escherichia coli* expression of Ab fragments. One involves the functional expression of correctly folded Ab fragments by secretion into the periplasm, where the oxidizing environment permits proper disulfide bond formation [59,60•]. This method is preferable if the scFv expressed is obtained at high yields of functional molecule, but it does not always achieve this result with proper folding of periplasmic protein being dependent on temperature, growth physiology, bacterial host, the presence of chaperonins, and the primary sequence of the expressed protein [59,60•,61]. An alternative strategy involves *in vitro* refolding of protein obtained either from intracellular inclusion bodies formed in the cytoplasm or from extracellular inclusion bodies formed in the periplasmic space [59,62•]. Solubilization and refolding procedures are then required to obtain functional protein, and the success of these manipulations varies depending on the particular Ab derivative: some scFv molecules may require very simple refolding procedures from inclusion bodies to recover a high yield of functional product whereas others require more complicated manipulations with low recoveries. A third strategy, which may involve either intracellular or extracellular localization, creates gene fusions of Ab fragments with bacterial or phage proteins in order to increase expression and stability [12•,63•], and subsequent cleavage of the carrier portion may be required for the scFv to be useful. A variety

of different vector systems and secretory signal peptides have been developed for the expression of Ab fragments in/from *E. coli* and its phage [12•,63•]. Again, the success of these methods depends to a great extent on the particular Ab fragment being expressed. It should be noted that for Ab fragments produced from phage display combinatorial libraries, the selection process eliminates those fragments which do not express or fold properly in *E. coli*, thereby improving the likelihood of successful bacterial expression.

Ab fusion proteins have also been expressed in functional form from insect cells such as *Drosophila* S2 and *Spodoptera* cells using baculovirus expression systems [64–66]. In one set of studies, baculovirus and myeloma expression of an anti tumor single chain Ab, CC49, and its IL-2 fusion protein, were compared [65]. The binding properties and functional activity of the mammalian and baculoviral derived proteins were identical. Both types of molecules were *N*-glycosylated and resistant to Endonuclease H digestion, yet no studies were performed to determine whether the glycosylation patterns of these fusion proteins differed sufficiently to alter their immunogenicity *in vivo*. Compared with myeloma cells, the insect cells produced 3- and 10-fold higher levels of the single chain Ab and its IL-2 fusion protein respectively, indicating that if immunogenicity and biodistribution/half-life are unaffected, baculovirus expression may be a viable alternative for the production of therapeutic Abs.

Another exciting development utilizes plant expression for recovering intact, complex Ab molecules [67••,68,69•]. One novel use of transgenic tobacco plants has permitted the recovery of stably assembled secretory Abs composed of IgA dimers and secretory component, a result difficult to achieve even in mammalian cell culture systems [67••]. Oral Ab delivery through foods such as edible transgenic plants might eliminate the need for purification and achieve localization of Ab to the gastrointestinal tract [67••,68]. Such molecules could be extremely useful for other therapies involving topical mucosal administration. One potential drawback to plant produced Ab derivatives is the difference in glycosylation that may alter their immunogenicity or half-life. More studies involving the systematic examination of several scFv-fusion proteins expressed in mammalian, baculoviral, yeast, plant, and bacterial systems are required to determine the optimal expression method for most Ab fusion constructs.

In addition to the production of soluble secreted molecules, many recent applications of Ab engineering involve specific compartmental expression of scFv fusion proteins within a target cell. Transfection of cells for intracellular targeting of scFv fragments can be achieved by various techniques [9•,41•], but for eventual gene therapy applications of such targeted scFv fusions, viral or retroviral transfection seems to be the most promising [9•,18•,33,36•,40•]. Targeting Abs to the cytosol or another intracellular compartment of the desired cell has been achieved by removing the Ab leader sequence, fusing the scFv to specific localization sequences, incorporating the fusion into an expression vector compatible with the target cell type, and transfecting the recombinant vector into target cells [9•,41•,70•]. These recombinant Ab molecules may interfere with the normal function of the target antigen and thereby alter the cell phenotype, as shown by Deshane *et al.* [36•,40•,71,72] using adenovirus-mediated expression of an endoplasmic reticulum (ER) targeted anti-erbB-2 scFv in erbB-2-expressing tumor cells. Expression of the scFv downregulated the cell surface expression of this tyrosine kinase receptor, suppressed proliferation, and ultimately triggered apoptosis in the target cells. In another set of studies [18•,33], scFv expression was targeted to the cell surface via fusion to the cytoplasmic and transmembrane domains of a cell surface receptor molecule. The cell surface forms of CD28-specific scFv expressed on several different cell types were found to function as artificial ligands for the CD28 T cell surface receptor, inducing costimulation of CD28-positive T cells.

Strategies for reducing immunogenicity

Many useful antihuman Ab specificities have been isolated from mouse hybridomas and are therefore immunogenic in humans. To be able to utilize these molecules as successful therapeutic agents, they must be redesigned to reduce their immunogenicity and thereby reduce the human-anti-mouse (HAMA) immune response. Chimerization, humanization, CDR grafting, and veneering are all methods which attempt to decrease immunogenicity while maintaining the binding specificity and affinity of the original Ab. Chimerization fuses unmodified variable domains to the constant regions of the light and heavy chains of a human antibody, usually κ and IgG1 respectively. Humanization involves not only the fusion of the (usually murine) variable domains to human constant regions, but also the alteration of selected murine variable domain framework residues to more closely match the most related available human variable template sequence. CDR grafting involves the substitution of non-human CDR domains from an antibody into the most closely related human antibody sequence available, so that only the CDR domains are non-human in origin. Veneering involves changing only exposed residues so that they reflect the human consensus sequences, while those residues which are buried are left unmodified. For reviews of these approaches, the reader is referred to two publications [73,74].

With the improved techniques for molecular modeling and the use of human frameworks which are the most homologous to the murine V region of interest, these strategies may often preserve the binding affinity of the original murine Ab while reducing immunogenicity significantly [75–77]. Many of these approaches, however, have been laborious, only to result in reduced antigen-binding affinity [78,79], and have failed to provide a rapid, reliable method for the generation of human Abs for immunotherapy. A

recent report by Rosok *et al.* [80•] uses combinatorial library strategies for rapid and selective humanization of murine Abs and for the isolation of clones with optimal specificities from large pools of mutants, so suggesting that iterative methods for humanization can be streamlined and refined.

Methods are being developed for the generation, selection, and isolation of human Abs to obviate the need for Ab humanization. The human humoral immune system is capable of generating huge numbers of diverse Ab-encoding genes, expressing these gene products on the surface of B lymphocytes, and mediating the antigen-driven selection of high-affinity Abs leading to Ab affinity maturation. Although it has proven difficult to generate human Abs by traditional hybridoma technology because of the difficulty in obtaining stable human hybridomas expressing high levels of these Abs, new developments in phage display of human Ab repertoires may permit the isolation of human Ab fragments without the problems associated with their murine counterparts [11,81,82]. The problems include not just immunogenicity but also attempting to preserve the stability and high avidity binding in a molecule not supported by the native Ab structure but selected within that context. These new approaches select for the desired properties within the context of the scFv and phage displayed material, so that the molecules are preselected and optimized for this mode of expression.

Phage display of Ab combinatorial libraries not only creates the direct physical linkage between genotype and phenotype that exists for B lymphocytes, but can also mimic *in vitro* many of the *in vivo* processes which result in the production of high-affinity Abs. Gene repertoires encoding Ab subunits can be created by the use of PCR and recombinant techniques to produce the fusion cassettes necessary for Ab subunit gene expression. Human Ab libraries have been constructed using preimmunized donors as the source of V region sequences [12•,14], and also using a defined subset of human germline V regions with either designed or randomized peptides inserted into one or more of the CDR regions [14,83], but usually in CDR3 [84]. These libraries can be expanded in complexity by the use of the phage P1 Cre/loxP site-specific recombinase system developed by Griffiths *et al.* [11] which exchanges light and heavy chain partners by recombination *in vivo*. Cre-mediated recombination between plasmid-borne V_H and phage-borne (V_L + dummy V_H) domains creates a V_L-V_H library of inserts with 6.5×10^{10} unique members, a level of diversity comparable to that of the *in vivo* human immune repertoire. A similar approach to generate highly diverse combinatorial libraries using the λint site-specific recombination system for cointegrate formation has been developed by Geoffroy *et al.* [85]. The purpose of both the Cre and λint systems is to achieve chain shuffling using site specific recombination mediated by a phage encoded recombinase without having to perform sequential library constructions, and so long as the cointegrate forms remain relatively stable, the two systems should be interchangeable.

Ab derivatives with the desired antigen specificities may be selected from phage display libraries by adhesion to purified immobilized antigens [11,86] or by selective binding strategies using unpurified cell-bound antigen [16••]. Clever techniques have been developed that couple antigen binding with phage infectivity and replication, mimicking the antigen driven selection and stimulation of B cells [15]. Nevertheless, artificial affinity maturation of phage display Ab repertoires has been achieved using error prone PCR, chain shuffling [87], random or directed mutagenesis of CDR regions [88–91], and sequential cycling of repertoires through a bacterial mutator strain until the desired affinity is reached [92]. There have been reports of achieving the nanomolar to picomolar range affinities desirable for therapeutic use by utilizing some of these techniques [11,14,93].

In addition to isolating high-affinity human Ab fragments targeted against foreign antigens, phage display systems can be used to isolate human antiself Abs with useful binding specificities [82,93]. Phage-displayed Ab fragments specific for human cell surface receptors have been selected by competitive elution with a receptor-specific mAb [94]. Recently, De Kruif *et al.* [16••] developed a strategy using flow cytometry for the direct selection of particular specificities for human antigens expressed on the surface of subpopulations of cells present in a heterogeneous mixture. This report is the first demonstration of a selective approach based on binding to unpurified antigen in a complex mixture of cells, a remarkably powerful extension of this type of technology. Artificial affinity maturation may not reproduce the mammalian affinity maturation mechanisms that could be important in eliminating Ab cross-reactivity with other self antigens *in vivo* or that regulate the structural properties of Abs to prevent immunogenicity. In a study which may relate to normal affinity maturation, Ohlin and Borrabaeck [95] analyzed repertoires derived from phage display and human *in vivo* Abs and found that the two repertoires differed in V_H and J_H segment usage and in heavy chain CDR3 length, indicating that the phage display derived Abs represent a more random selection of the Ab repertoire than that obtained from *in vivo* human immune responses. Although these issues need to be explored further, the rapidity and efficiency with which phage display Abs can be generated and selected is unmatched by other techniques currently available.

Another novel approach to the production of completely human mAbs is the creation of transgenic mice defective in the production of mouse Abs [96] and engineered to produce a large repertoire of human Abs [97–101,102•]. Gene targeting technology has been used to delete *cis*-acting sequences important for gene rearrangement

and expression in embryonic stem (ES) cells, thereby eliminating mouse Ab production [96,103,104]. Yeast artificial chromosomes (YACs) were then constructed containing large fragments of the human heavy and light chain Ig loci, and introduced into the ES cells by fusing them with YAC-containing yeast spheroplasts [96,103–105]. An alternative method used to engineer Ab production involves the use of human Ig minigenes, which fuse noncontiguous genomic segments of V and constant (C) regions from the human Ig loci. The fused genomic segments are then transferred by microinjection into mouse pronuclei [98–100,104,106]. Both of these approaches have successfully generated transgenic mice expressing human Igs, demonstrating that the human genes can be properly rearranged and expressed, can undergo somatic mutation, and can engage in isotype switching in the mouse [96,98,104]. These animals, however, lack complete B-cell reconstitution, possessing lower levels of mature B cells in lymphoid organs and a partial block in the pro- to pre-B-cell transition in the bone marrow [96,98,104]. In addition, the pattern of VDJ recombinations observed for the minigene approach is characteristic of human fetal Ig rearrangements [97,107], while that for the YAC approach more closely reflects normal adult human B cell patterns of rearrangement [96]. These partial phenotypes might be corrected by replacement of the mouse Ab sequences by larger and more complete repertoires from the human Ig loci.

Like all mice, the transgenic mice lack tolerance to human protein antigens, except those encoded by the transgenic human Ig loci. High-affinity human anti-human Abs can therefore be isolated from transgenic mice after immunization with human antigens [98,104]. Hybridoma cell lines can then be derived from fusions of mouse myeloma cells with splenocytes from the immunized transgenic mice [98,104]. By generating high-affinity human mAbs directed against human antigens, transgenic mice and phage display of human Ab repertoires may eventually eliminate the need for humanization techniques.

One other recent report [108••] describes the use of *in vitro* immunization of naive human B cells using antigen specific T helper cells, CD40 ligation, and antigen. Secondary rounds of *in vitro* immunization with these reagents generated high-affinity human Abs which had undergone isotype switching. The Ig cDNA from these cells was cloned, amplified by PCR, and inserted into phage display libraries that link replication and recognition functions, thereby preferentially amplifying higher affinity clones. Many other groups have attempted to achieve such high-affinity Ab production *in vitro* with little success beyond producing low-affinity Abs, usually IgM, which have not undergone switching. If the described *in vitro* immunization approach proves to be reliable and reproducible, it may also provide a future source of high-affinity human Ab genes.

Bispecific gene fusions

Bispecific molecules possess two unique functions or binding specificities within a single molecule and have tremendous potential for applications, including immunoassays, immunohistochemistry, cell targeting, immunomodulation, targeted delivery of cytokines or effector functions, and tumor therapy. In general, bispecific molecules that include at least one Ab domain are produced either by recombinant techniques involving direct gene fusions of the two protein domains or by engineering sequences for self-assembly onto individual gene cassettes. ScFv molecules can also be fused at either the amino- or carboxy-terminal ends, permitting functional joining with extracellular domains of membrane receptors, toxins, intracellular localization sequences, or other scFvs.

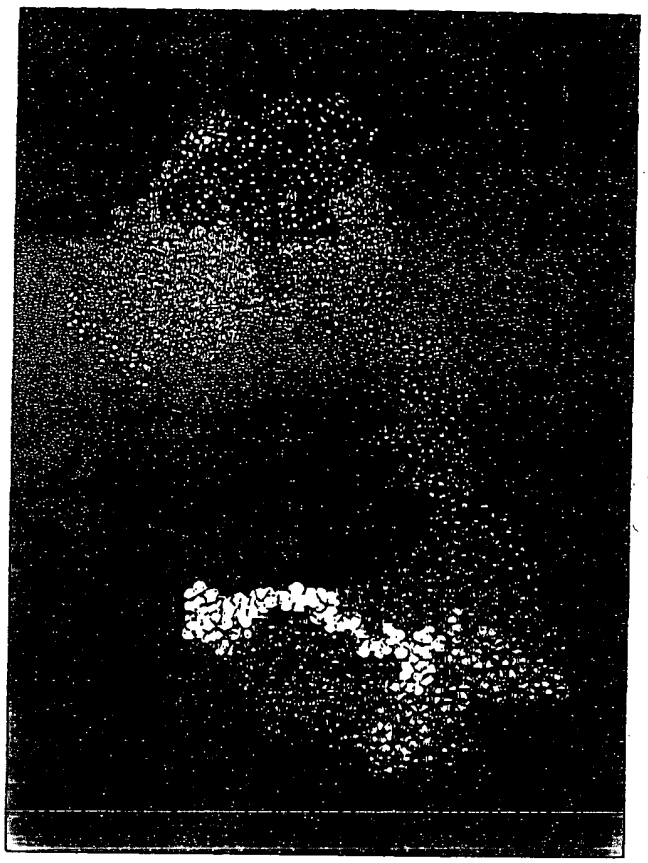
Recombinant immunotoxins are chimeric proteins which fuse an scFv or Fab to a truncated plant or bacterial toxin domain, and have provided an alternative to chemical conjugates as targeting agents for tumor therapy [21,22]. These fusion proteins show more effective tumor penetration and more rapid clearance from the circulation than the chemical conjugate immunotoxins, due to their small size [20–22]. The toxin portion and possibly the scFv portion of the scFv immunotoxins may be immunogenic, however, limiting the clinical utility of immunotoxin bispecific fusion proteins for chronic or long-term therapies. As an alternative, to address the problem of toxin immunogenicity, genetic immunotoxins fuse the Ab domain to a DNA-binding protein bound to an expression plasmid containing the toxin expression cassette [109•,110]. For example, the positive charges of human protamine, a DNA-binding protein, can strongly interact with the negative charges of DNA, generating a neutral, stable, much less immunogenic fusion partner for the Ab than the toxin protein. After internalization of the Ab–plasmid complex by receptor-mediated endocytosis, expression of the toxin results in cell killing. More selective expression of the toxin can be achieved by placing the expression cassette under the control of inducible or cell-specific promoters. This overcomes the problem of incomplete specificity caused by the Ab domain binding to cells, other than those of specific interest, expressing the target antigen. Such an approach might prove to be particularly powerful at maximizing tumor specific killing while minimizing toxic side effects and immune reactivity generated against the therapeutic agent.

Bispecific Abs have been produced by three principal techniques: chemical conjugation of two Ab molecules [28,111], fusion of two different hybridoma cell lines to create hybrid hybridomas [26,30,112,113], and genetic manipulation of recombinant molecules [24,25,27,31–33,35]. Production by the first two approaches generates a heterogeneous mixture of different products, requiring labor intensive methods to purify the bispecific form. Re-

placement of these traditional methods with recombinant approaches to derive bispecific molecules has accelerated progress in this area. One recent report [114] of 'knobs into holes' describes engineering sequence changes into the C_H3 domains of heavy chains so that formation of bivalent homodimers (Ab1–Ab1; Ab2–Ab2) is less favored than heterodimerization between Ab1-knob C_H3 + Ab2-hole C_H3. Within the past two years, there have been numerous reports characterizing bispecific Ab derivatives *in vitro* and *in vivo* [24–35]. Each of these reports uses unique combinations of Ab derivative structure and expression systems to achieve production of functional, bispecific Abs. Most of these molecules are designed to retarget cytotoxicity or immune recognition functions to tumor cells, although a few reports have involved the generation of molecules for use in immunoassays or imaging. Some bispecific Abs are generated by constructing single chain molecules, composed of linked variable chain domains with differing specificities, fused to human Fc domains [24,33,35]. Figure 2 shows a molecular model of an αCD3–αL6FvIg bispecific fusion protein which our group has generated [24] using this approach. This molecule mediates strong T cell activation and cytotoxic responses against L6-expressing tumors *in vitro*. Despite their size, which is larger than a bare scFv or a diabody type structure, these molecule forms possess the longer serum half-life preferred for stimulating strong anti tumor responses *in vivo*.

Another approach to generating bispecific Ab fragments utilizes the diabody method developed by Holliger *et al.* [5]. Briefly, the diabody method constructs scFv chains with short linkers, which prevents intrachain pairing but allows interchain pairing to form bivalent fragments. If the scFvs contain V_L and V_H for two different antigen targets, then assembly with the reciprocal scFv pair can generate bispecific Ab fragments. McGuinness *et al.* [115•] have recently developed a phage display diabody approach to generating and screening bispecific Ab scFvs. Zero linker diabody libraries were constructed with the following configuration in the phagemid vector pCANTAB6: rbs-leader1-V_HA-V_LB-STOP-rbs-leader2-V_HB-V_LA-His6-myc-amber-fdgene3. The constituents of the vector all play important functions. The ribosome binding site (*rbs*), a short sequence of bases preceding the actual coding region, is important for achieveing good translation of transcribed messages in bacteria. The leader peptide sequences are important for the export of the nascent proteins from the cell. The His6 tag is present for protein purification using gentle affinity chromatography, and the myc tag is present to provide a domain distinct from the scFv regions detectable by antibody probes. The amber codon provides a method for manipulating attachment of the gene 3 protein to the carboxy terminus of the diabody. In a suppressor strain of bacteria the gene 3 protein will be attached, while in a nonsuppressor strain the reading frame will stop at the amber codon thereby eliminating the gene 3 tail from the protein.

Figure 2



Model of a bispecific scFv composed of the G19-4 (anti-human CD3 or αCD3) and L6 (anti-human L6 or αL6) V regions. The CDRs of the G19-4 scFv (V_L plus V_H) are shown in purple, and the CDRs of L6 scFv (V_L plus V_H) are shown in red. The helical linker between the scFvs is shown in blue-green while the flexible $(Gly_4\ Ser)_3$ linker is shown in very pale green (in the bottom part of the figure) for the G19-4 scFv and in green for the L6 scFv.

If Ab selection is required, diabodies can be displayed on phage and selected on antigen; alternatively, soluble diabody molecules can be produced using nonsuppressor bacteria. Although the diabody approach appears promising with previously cloned scFv molecules, construction of diabody libraries from large Ab repertoires requires many cloning steps, the selection methods may prove problematic, and the library stability in bacteria has not yet been adequately tested to determine how useful a system this will be for construction and selection of optimal bispecific scFvs.

An alternative strategy utilizes leucine zipper motifs to induce self-assembly of bispecific miniAbs [116–118,119•]. A recent report by de Kruif and Logtenberg [119•] describes the fusion of Fos or Jun leucine zippers and a truncated mouse IgG3 upper hinge region to

scFv proteins. Two cysteine residues were engineered into the zipper domains to produce disulfide-stabilized homodimers. Utilizing scFvs isolated from a phage display library and these modified zipper cassettes, de Kruif and Logtenberg produced functional stable secreted homodimers in *E. coli*. *In vitro* reduction, mixing, and reoxidation of the Fos and Jun scFv Abs resulted in the production of functional bispecific $(scFv)_2$ molecules. The authors failed to address, however, methods for the efficient, rapid and quantitative purification of heterodimers and the removal of contaminating homodimers, an important step in making this strategy more generally useful. Rheinnecker *et al.* [120] utilized a similar approach to create scFv dimers or oligomers by fusion of an artificial helix-turn-helix dimerization domain or the tetramerization domain of p53 respectively to a long IgG3-like hinge–scFv construct. In the future, novel bispecific molecular forms will continue to be generated by applying domain shuffling approaches such as those described here.

Small antigen-binding fragments

Rapid developments have occurred in the past few years in the area of peptide mimetics, or small antigen-binding fragments that mimic the antigen specific binding activity of an Ab or natural ligand. Again, this progress is due in large part to the application of new techniques such as BIAcore® analysis of binding kinetics. BIAcore® refers to real-time bimolecular interaction analysis (BIA). This method utilizes the optical phenomenon of surface plasmon resonance to monitor interactions between biomolecules on a sensor surface [121,122]. Detection relies on changes in refractive index close to the surface which occur as analyte from solution binds to the immobilized binding partner, the ligand. Measurements are made in real time so that both kinetic and affinity data can be extracted from the same experimental runs. Other new techniques include combinatorial chemistry libraries phage display libraries of peptides or protein subdomains, computer aided modeling of peptide and protein structures, and improvements in structure determinations using X-ray crystallography and nuclear magnetic resonance (NMR). Chemically synthesized and biologically generated peptide libraries have been used to isolate peptide ligands for several receptors and Abs [123,124]. Introduction of the appropriate conformational constraints into the antigen binding fragments can facilitate binding interactions due to decreases in the loss of conformational entropy associated with antigen binding [125]. In the case of Ab mimetics, the canonical structure can be predicted by sequence analysis and computer modeling and the appropriate target conformation locked by the introduction of chemical constraints on the peptide [126]. For example, Levi *et al.* [126] constructed six peptides composed of the CDRs from an anti-HIV-1 Ab and determined their activity against the viral envelope protein. CDR_H3 was the most effective in preventing syncytia formation in infected cells, and cyclization of this peptide resulted in improved inhibition of both syncytia formation and binding of the parent Ab as compared to the linear form.

Until recently, all of the small antigen-binding fragments isolated functioned as antagonists, disrupting the normal binding interactions of the target antigen or Ab. In one of the more elegant applications of these new technologies, small peptide mimetics of the hormone erythropoietin (EPO) were isolated using a soluble form of the EPO receptor (EpoR) to screen random DNA sequence phage display libraries encoding eight-residue peptides as fusions to the bacteriophage fd coat protein pVIII [127••]. The peptide-encoding sequences were flanked by codons encoding cysteine residues in order to create constrained, intramolecular disulfide-bonded cyclic peptides from these libraries. Once the binders to the EpoR were isolated from the initial library, phagemid display mutagenesis was performed using the bacteriophage M13 coat protein pIII as the display vehicle, and further random residues were added to the original octamer peptides in order to recover high-affinity binders. These high-affinity binders were selected by the use of EPO as a competitor for binding to the EpoR under conditions of increasing stringency. These higher affinity peptides were not homologous to the primary sequence for EPO, yet were capable of binding to and activating the EpoR. This is one of the first demonstrations of success in isolating agonist forms of peptide mimetics.

Conclusions

Recent advances in several areas of research have facilitated the rapid progress in Ab engineering. Advancements in the understanding of Ab structure and antigen–Ab interactions have been made through assembly of large sequence databases, computer modeling, structural analysis including X-ray crystallography, NMR, site-directed mutagenesis, and surface plasmon resonance analysis of antigen–Ab binding kinetics. Increasingly, recombinant techniques for cloning and manipulating Ab derivatives are leading to the development of rapid, reliable methods for generating the desired Ab molecules. Continual progress is also being made in developing large-scale expression systems for synthesizing Abs and their derivatives, with the goal of maximizing cell density, culture longevity, and secretion productivity, while minimizing the number of downstream purification steps and other manipulations. Similar progress has also been made in developing expression systems designed for the rapid screening of new Ab molecules or the ectopic expression of Ab subunits in intracellular compartments. Selective approaches which permit the isolation of desired specificities from huge combinatorial libraries using advances in phage display technology have revolutionized the field of Ab engineering and the development of peptide mimetics. Future developments in Ab engineering may eventually result in fulfillment of the tremendous potential of Abs for therapeutic and diagnostic applications.

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KSA Antigen Ep-CAM Mediates Cell–Cell Adhesion of Pancreatic Epithelial Cells: Morphoregulatory Roles in Pancreatic Islet Development

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stract. Cell adhesion molecules (CAMs) are important mediators of cell–cell interactions and regulate cell fate determination by influencing growth, differentiation, and organization within tissues. The human pancarcinoma antigen KSA is a glycoprotein of 40 kD originally identified as a marker of rapidly proliferating tumors of epithelial origin. Interestingly, most normal epithelia also express this antigen, although at lower levels, suggesting that a dynamic regulation of KSA may occur during cell growth and differentiation. Recently, evidence has been provided that this glycoprotein may function as an epithelial cell adhesion molecule (Ep-CAM). Here, we report that Ep-CAM exhibits the features of a morphoregulatory molecule involved in the development of human pancreatic islets. We demonstrate that Ep-CAM expression is targeted to the lateral domain of epithelial cells of the human fetal pancreas, and that it mediates calcium-independent cell–cell adhesion. Quantitative confocal immunofluorescence in fetal pancreata identified the highest levels of Ep-CAM expression in developing islet-like cell clusters budding from the ductal epithelium, a cell compartment thought to comprise endocrine progenitors. A surprisingly reversed pattern was observed in the human adult pancreas, displaying low levels of Ep-CAM in islet cells and high levels in ducts. We further demonstrate that culture conditions promoting epithelial cell growth induce upregulation of Ep-CAM, whereas endocrine differentiation of fetal pancreatic epithelial cells, transplanted in nude mice, is associated with a downregulation of Ep-CAM expression. In addition, a blockade of Ep-CAM function by KS1/4 mAb induced insulin and glucagon gene transcription and translation in fetal pancreatic cell clusters. These results indicate that developmentally regulated expression and function of Ep-CAM play a morphoregulatory role in pancreatic islet ontogeny.

INDUCTION and maintenance of tissue differentiation during development depends on the coordinated spatiotemporal expression of specialized molecules that regulate cell-to-cell and cell-to-matrix interactions (Ekblom et al., 1986; Edelman, 1991, 1992; Takeichi, 1991, 1995; Trelstad, 1984). Since the pioneering work of Holtfreter (1939) and Moscona (1952), who first recognized the existence of cell type–specific adhesive properties in multicellular organisms, the functional portrait of cell adhesion molecules (CAMs)[1] has evolved from that of simple "binding molecules" to the modern concept of "morphoregulatory molecules." In fact, their coordinated action appears to be involved in the regulation of cell growth, differentiation, adhesion, migration, and three-dimensional organization within tissues during morphogenesis (Crossin et al., 1985; Ekblom et al., 1986; Edelman et al., 1991; Edelman, 1992; Takeichi, 1991, 1995).

An exquisite example of timely regulated morphogene-

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1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; DS, donkey serum; Ep-CAM, epithelial cell adhesion molecule; HFP, human fetal pancreas; ICC, islet-like cell cluster; N-CAM, neuronal cell adhesion molecule; rhGHF/SF, recombinant human hepatocyte growth factor/scatter factor.

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sis is provided by the cell growth, differentiation, and organization of pancreatic islets of Langerhans, representing the endocrine compartment of mammalian pancreas (Langerhans, 1869; Orci and Unger, 1975; Orci, 1982). It is currently thought that islet cells originate from undifferentiated progenitors resident within the ductal epithelium of the fetal pancreas (Pictet and Rutter, 1972; Teitelman and Lee, 1987; Alpert et al., 1988; Herrera et al., 1991; Gu and Sarvetnick, 1993). This process involves cell budding, growth, migration into the surrounding mesenchyme, and differentiation into the highly organized islet clusters (Pictet et al., 1972; for review see Slack, 1995). Evidence has been provided for a role of adhesion molecules of the cadherin family in the morphogenesis of the pancreas (Thiery et al., 1982, 1984; Edelman et al., 1983; Hatta and Takeichi, 1986; Nose and Takeichi, 1986; Levi et al., 1991; Sjödin et al., 1995), and in the development of islet clusters (Dahl et al., 1996). Similarly, adhesion molecules of the immunoglobulin superfamily such as neuronal (N)-CAM have been found dynamically expressed in the pancreas and in other organs of endodermal origin during development (Edelman et al., 1983; Rutishauser, 1984; Crossin et al., 1985). In addition, we and others have demonstrated the involvement of cadherins and N-CAM in islet cell–cell adhesion (Langley et al., 1989; Begemann et al., 1990; Rouiller et al., 1990, 1991; Bauer et al., 1992; Moller et al., 1992), and the regulation of islet cell types' organization by calcium-independent adhesion molecules such as N-CAM (Rouiller et al., 1991; Cirulli et al., 1994).

Among the molecules possibly involved in tissue morphogenesis, the pancarcinoma antigen KSA (alias EGP40, 17-1A, ESA, etc.) is particularly interesting (Varki et al., 1984; Edwards et al., 1986; Spurr et al., 1986; Bumol et al., 1988). This antigen, originally identified as an abundantly expressed glycoprotein in tumors of epithelial origin, is found at lower levels in most simple, pseudostratified and transitional normal epithelia (Moldenhauer et al., 1987; Momburg et al., 1987). Fetal epithelia exhibit stronger immunoreactivity for KSA antigen than the adult mature tissues (Varki et al., 1984), suggesting a dynamic regulation of its expression during epithelial ontogeny. Recently, Litvinov and colleagues provided evidence that EGP40 (alias KSA) exhibits the features of a typical cell–cell adhesion molecule when transfected in murine and human tumor cell lines (Litvinov et al., 1994*a*,*b*). The name of Ep-CAM (epithelial cell adhesion molecule) for this antigen was therefore proposed.

To identify novel morphoregulatory molecules possibly involved in pancreatic islet development, we have investigated the expression and function of Ep-CAM in the developing human pancreas. We demonstrate that Ep-CAM expression is targeted to the lateral domain of epithelial cells of the human fetal pancreas, and that it mediates calcium-independent adhesion. In addition, we show that Ep-CAM expression is developmentally regulated during cell growth and endocrine determination of islet cells. Based on properties and roles ascribed to classical cell–cell adhesion molecules in histogenesis and organogenesis (Edelman and Crossin, 1991; Takeichi, 1995), we propose that Ep-CAM represents a novel morphoregulatory molecule delivering specific developmental signals at key stages of pancreatic islet morphogenesis.

Materials and Methods

Tissues

Midgestation human fetal pancreata (HFP) ranging from 18 to 20 wk of gestational age were obtained through nonprofit organ procurement programs (Advanced Bioscience Resources, Oakland, CA; Anatomic Gift Foundation, Laurel, MD), which also obtained consent for tissue donation. Samples of human adult pancreas and pancreatic islets were prepared at The Diabetes Research Institute (University of Florida, Miami, FL) as previously described (Ricordi et al., 1988). Use of human tissues was approved by appropriate Institutional Review Boards.

Immunofluorescence, Confocal Microscopy, and Morphometric Analysis

Triple immunofluorescent labeling for Ep-CAM, insulin, and glucagon, or Ep-CAM, Ki-67, and insulin were performed on 8-μm-thick cryostat sections prepared from snap frozen fetal (18–20 wk of gestation) and adult pancreases. Sections were mounted on glass slides, dried at room temperature for 1 h, fixed in freshly made 4% formaldehyde (from paraformaldehyde) for 20 min at 4°C, permeabilized in 0.1% saponin for 5 min at room temperature, and then incubated in 50 mM glycine in PBS to saturate reactive groups generated by formaldehyde fixation. Nonspecific binding was blocked by incubation of sections in PBS containing 2% donkey serum (DS) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and 1% BSA fraction V (Sigma Chemical Co., St. Louis, MO) for 1 h at room temperature. After extensive washes in PBS-DS (0.2% DS, 0.1% BSA, 5 mM glycine), sections were incubated for 1 h at room temperature with a mixture of primary antibodies: anti–Ep-CAM KS1/4 mAb (5 μg/ml) (Varki et al., 1984), IgG fraction of a sheep antiinsulin polyclonal antiserum (5 μg/ml; The Binding Site, Birmingham, United Kingdom), rabbit antiglucagon polyclonal antiserum (1:200 dilution; Chemicon International, Inc., Temecula, CA) or rabbit anti–Ki-67 affinity-purified IgGs (2.5 μg/ml; Dako Corp., Carpinteria, CA). In separate sections, a mixture of normal sheep, rabbit, and mouse IgGs was used as control reference for specificity of primary antibodies. After several washes in PBS-DS, sections were incubated for 1 h at room temperature with a cocktail of $F(ab')_2$ fractions from secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.): lissamine rhodamine–conjugated affinity-purified donkey anti–sheep IgGs (5 μg/ml) (preadsorbed on chicken, guinea pig, hamster, horse, human, mouse, rabbit, and rat serum proteins); FITC-conjugated affinity-purified donkey anti–rabbit IgGs (5 μg/ml) (preadsorbed on bovine, chicken, goat, guinea pig, hamster, horse, human, mouse, rat, and sheep serum proteins); indodicarbocyanine (Cy5)-conjugated affinity-purified donkey anti–mouse IgGs (5 μg/ml) (preadsorbed on bovine, chicken, goat, guinea pig, hamster, horse, human, rabbit, rat, and sheep serum proteins). After six washes at 5 min each with PBS-DS, sections were mounted in slow fade medium (Molecular Probes, Inc., Eugene, OR), and then viewed on a microscope (model Axiovert 35M; Carl Zeiss, Inc., Thornwood, NY) equipped with a laser scanning confocal attachment (model MRC-1024; Bio-Rad Laboratories, Hercules, CA), using a 40× 1.3 NA objective lens. Fluorescent images relative to each marker were collected by using the 488-, 568-, and 647-nm excitation lines from an argon krypton mixed gas laser. Color composite images were generated using Adobe Photoshop 4.0 (Adobe Systems Inc., Mountain View, CA) running on a computer (model SuperMac S910/250; UMAX Computer Corp., Fremont, CA), and then printed with a Fujix Pictrography 3000 color printer (Fuji Photo Film U.S.A., Inc., Elmsford, NY).

Microscopic fields acquired from fetal ($n = 50$) and adult ($n = 72$) pancreatic sections immunostained for Ep-CAM, insulin, and glucagon were analyzed for pixel intensity of Ep-CAM–specific immunoreactivity using National Institutes of Health (NIH) Image software (Bethesda, MD). Pixel's intensity units (0–255) were recorded from a total of 250 domains of cell–cell contact for each cell type (ductal, endocrine, epithelial undifferentiated, and exocrine) in both fetal and adult pancreatic specimens.

Immunoelectron Microscopy

IIF³ cells cultured as monolayers (Beattie et al., 1996) were fixed with 4% formaldehyde in PBS for 30 min, washed in PBS, and then permeabilized with a solution containing 0.1% Triton X-100 and 2% normal goat serum in PBS for 20 min. Cells were incubated with primary antibodies (KS1/4 mAb or anti-EcadEC5 polyclonal antibody [Fannon et al., 1995]) for 1 h, washed in buffer, and then incubated with biotinylated goat anti-mouse

or anti-rabbit IgG for 1 h at 4°C. After washes in PBS, the cells were incubated in an avidin–biotin complex for 1 h, washed again in PBS, and then reacted for 4–6 min in 0.05 mg/ml diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) and 0.01% H_2O_2 in PBS. The cells were then post-fixed with 1% OsO_4 in PBS for 1 h, rinsed in distilled water, dehydrated in an ethanol series, and then embedded in Durcupan resin (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin (80-nm) sections and semithin (1-μm) sections were cut using a diamond knife and an ultramicrotome (model Ultracut E; Leica USA, Deerfield, IL) and then mounted on uncoated copper grids. Thin sections were imaged at 80 keV using an electron microscope (model 100CX or 2000FX; JEOL Ltd., Akashima, Japan) and then semithick sections were imaged at 300–400 keV using an intermediate voltage electron microscope (model 4000EX; JEOL Ltd). Stereo pair electron micrographs were obtained by tilting the specimen ±5°.

Generation of Islet-like Cell Clusters and Cell Monolayers

HFP at 18–24 wk of gestation were minced in small pieces and then digested in a collagenase solution (6 mg/ml per HBSS; collagenase-P; Boehringher Mannheim Biochemicals, Indianapolis, IN) for 15 min at 37°C in a shaking water bath (150 cycles/min) as described previously (Otonkoski et al., 1993). Cell clusters resulting from this procedure were washed in cold HBSS and then placed in culture in RPMI-1640 (Sigma Chemical Co.) containing 11 mM glucose and supplemented with 10% normal human serum. This culture condition, applied for 3–5 d in nonadherent culture dishes, allows the formation of smooth islet-like cell clusters (ICCs). ICCs contain mostly undifferentiated epithelial cells (Beattie et al., 1994; Otonkoski et al., 1994, 1996) and between 5 and 10% endocrine cells (mainly insulin- and glucagon-producing cells). Cells within ICCs can be induced to proliferate in vitro to threefold when cultured in the presence of 10 ng/ml of recombinant human hepatocyte growth factor/scatter factor (rhHGF/SF) (Otonkoski et al., 1994; Beattie et al., 1996). Cultures of ICCs in Petri dishes coated with the extracellular matrix 804G, as recently described by Langhofer and co-workers (Langhofer et al., 1993), were supplemented with 10 ng/ml rhHGF/SF, and then used to generate cell monolayers. Under these culture conditions, cells forming the ICCs adhere, migrate, and proliferate to produce large monolayers that result from a 30-fold increase in cell number (Beattie et al., 1996). Cell monolayers prepared in this manner were therefore used as an in vitro model of pancreatic epithelial cell growth.

Transplantation Experiments and Glucose Tolerance Test

Four athymic nude (nu/nu) BALB/c mice were transplanted under the kidney capsule with 500 ICCs/mouse, as previously described (Sandler et al., 1986; Beattie et al., 1994). At the time of transplant, ICCs contain ~5–10% endocrine cells, <10% mesenchymal cells, and ~80% undifferentiated epithelial cells (Beattie et al., 1994), whereas after 12 wk in vivo, ~80% of the grafted cells have differentiated into endocrine tissue (Beattie et al., 1997). Therefore, we used this in vivo model to study the expression pattern of Ep-CAM during islet cell development from immature pancreatic epithelial cells. After 12 wk, the mice were fasted for 4 h, anesthetized, and then blood samples were collected from the external jugular vein before and 30 min after an intraperitoneal injection of glucose (3 g/Kg of body weight). Blood samples were then tested for the presence of human C peptide using a human-specific radioimmunoassay, as previously described (Beattie et al., 1994).

Flow Cytomery

HFP were minced and digested in collagenase (3 mg/ml per HBSS; collagenase-P, Boehringher Mannheim Biochemicals) for 15 min at 37°C in a shaking water bath (150 cycles/min). Cell clusters resulting from this procedure were washed in cold PBS containing 0.2 mM EDTA, resuspended in a nonenzymatic cell dissociation medium (Sigma Chemical Co.) and then incubated at 37°C for 10 min applying gentle pipetting every minute. The single cell suspension resulting from this protocol was washed in HBSS containing 1% BSA (HBSS-BSA), and then incubated for 45 min at 4°C with either anti-Ep-CAM KS1/4 mAb (1 μg/ml) or isotype-matched control IgGs. After washing in HBSS-BSA, cells were incubated with FITC-conjugated donkey anti-mouse IgGs (H + L chains) for 45 min at 4°C. After washing in HBSS-BSA, samples were then analyzed on a FACScan™ flow cytometer (Becton Dickinson, Sparks, MD).

Production of Fab' Fragments

Control or anti-KSA (Ep-CAM) Fab' fragments were generated by using an immobilized papain digestion protocol (Pierce Chemical Co., Rockford, IL) with either 50 mg of control mouse IgGs or 50 mg of KS1/4 mouse mAb. After separation of Fab' fragments according to the manufacturer's directions, Fab's were concentrated by ultrafiltration using Centricon-10 units (Amicon Corp., Beverly, MA) and then resuspended in sterile PBS at a concentration of 1 mg/ml. Purity of Fab' fraction was checked by analyzing a sample of Fab' in a 10% agarose gel and followed by Coomassie blue staining. Reactivity of KS1/4 Fab' to Ep-CAM was confirmed by flow cytometry.

Cell Aggregation and Antibody Perturbation Assay

A single cell suspension was prepared from HFP, as detailed above, and used to test cell–cell aggregation mediated by "calcium-dependent" and "calcium-independent" adhesion systems. These pancreatic cells were divided into two pools and then washed in Krebs-Ringer bicarbonate buffer containing 1% BSA, 10 mM glucose, 10 mM Hepes, 50 μg/ml of DNase I, and either 1 mM $CaCl_2$ or 0.2 mM EDTA. Each of the two cell pools was resuspended at a concentration of 10^6 cells/ml and then further divided into two aliquots to test the ability of pancreatic cells to reaggregate either in the presence of a control Fab' fragment or in the presence of an anti-Ep-CAM Fab' fragment (100 μg/ml). Cell aggregation for each sample was performed in triplicate for 45 min at 37°C in a shaking water bath (100 cycles/min). Cell aggregation was monitored by counting the number of events in a particle counter (ZM-Coulter counter; Coulter Corp., Miami, FL) at the beginning and at the end of the aggregation period, as previously described (Rouiller et al., 1990, 1991; Cirulli et al., 1993).

Effect of Anti–Ep-CAM Fab' on Fetal Pancreatic Cell Differentiation

Single cell suspensions prepared from HFP, as detailed above, were plated in medium containing either anti–Ep-CAM KS1/4 mAb or control Fab' fragments (100 μg/ml). Fab's (50 μg/ml) were added to the cultures daily to ensure constant saturation of Fab's blocking activity. After 5 d in these culture conditions cells were harvested, and then total RNA was prepared to test insulin and glucagon transcript levels in an RNase protection assay as described (Otonkoski et al., 1993; Mally et al., 1994). In some experiments, samples of both control and anti–Ep-CAM–treated cells were used for DNA and insulin protein determinations (Otonkoski et al., 1993).

Immunoblotting

Samples of cultured ICCs, pancreatic cell monolayers, fetal and adult human islets, and human insulinomas were extracted in 20 mM Tris, 1% Triton X-100, pH 7.2, for 30 min at 4°C. Insoluble material was pelleted by spinning at 10,000 *g* for 20 min and then supernatants were recovered. Total proteins, 5 μg from each sample, were diluted in electrophoresis sample buffer, boiled for 5 min, and then subjected to 10% SDS–acrylamide gel electrophoresis. Proteins were then electrotransferred onto polyvinylene difluoride (PVDF) membranes (Millipore Corp., Waters Chromatography, Bedford, MA). After blocking in Blotto solution (Tropix, Inc., Bedford, MA), membranes were incubated for 2 h at room temperature with either KS1/4 mAb (1 μg/ml), or an isotype-matched mouse IgG. After extensive washings, PVDF membranes were incubated for 1 h at room temperature with a peroxidase-conjugated goat anti–mouse antibody (1:5,000 dilution). Antibody binding was revealed by a chemiluminescence detection system (KLM, Gaithersburg, FL) following the manufacturer's instructions.

RNA Isolation and Hormone Transcriptional Analysis

Total cellular RNA was isolated by the acid guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) and then quantitated spectrophotometrically. Transcriptional analyses were performed using a multiprobe RNase protection assay (Otonkoski et al., 1993). All probes used were of human origin and previously described (Otonkoski et al., 1993; Mally et al., 1994). Yeast tRNA (10 μg) was included as a negative control. Quantitation of band intensities was performed by scanning densitometry (model LKB UltroScan XL Laser; Pharmacia Biotech., Inc., Piscataway, NJ) and integrated using GelScan XL software (Pharmacia Biotech, Inc.). Different exposure times were used to ensure that all signals fell

within the linear range of the densitometer. The probe-specific mRNA signals were normalized to the cyclophilin signal in each sample to account for differences in sample loading between lanes.

Statistics

Where applicable, data were analyzed using Stat View 4.01 software (Abacus Concepts, Inc., Berkeley, CA) for determination of mean, standard deviation, and parametric statistics (*t* test).

Results

Ep-CAM Expression Highlights the Epithelial Compartment of the Developing Human Pancreas

In humans, the development of the endocrine compartment of the pancreas, the islets of Langerhans, occurs during fetal life, whereas differentiation, growth, and expansion of the exocrine acinar tissue is a perinatal event (Fukayama et al., 1986; Jaffe, 1991; Carrere et al., 1992). In all mammals studied, these two functionally distinct compartments are believed to derive from common precursors present in the ductal epithelium engaging separate differentiation programs at distinct developmental stages (Like and Orci, 1972; Pictet and Rutter, 1972; Pictet et al., 1972; Clark and Grant, 1983; Stefan et al., 1983; Teitelman and Lee, 1987; Alpert et al., 1988; Von Dorsche et al., 1989; Gu et al., 1994). At a midgestational age (16–20 wk), the epithelial compartment of the human pancreas consists of a branching ductal tree giving rise to numerous clusters of cells that appear to protrude and invade into the surrounding mesenchyme. To identify the expression pattern of Ep-CAM within the developing human pancreas, 8-μm cryostat sections from a human fetal pancreas of 18 wk were used for the simultaneous identification of Ep-CAM–, insulin- and glucagon-positive cells by triple immunofluorescence. As shown in Fig. 1, Ep-CAM–specific immunoreactivity (*A*, *green*) highlights the epithelial compartment of the fetal pancreas, allowing the identification of ducts and clusters of cells budding from the ductal epithelium (*arrowheads*). Many of these budding cell clusters contain insulin (Fig. 1 *C*, *red*) and glucagon-positive cells (Fig. 1 *B*, *blue*), a feature consistent with the current notion that endocrine cells arise from precursors present within the ductal epithelium (Pictet et al., 1972; Teitelman and Lee, 1987; Alpert et al., 1988; Gu and Sarvetnick, 1993). Notably, Ep-CAM expression appears to be restricted to sites of cell–cell contacts in a pattern similar to that observed for classical adhesion molecules, suggesting its involvement in the regulation of pancreatic epithelial cell–cell adhesion.

Ep-CAM Expression Is Targeted to the Lateral Domains of HFP Epithelial Cells

To gain insight on the distribution of Ep-CAM to specific cellular domains, cell monolayers prepared from HFP were immunolabeled for Ep-CAM by an indirect peroxidase method. Sections were cut either through a horizontal plane parallel to the cell monolayer, or through a vertical plane perpendicular to the monolayer, and imaged at 300–400 keV using an intermediate voltage electron microscope (JEOL 4000EX). A recently described anti–E-cadherin antiserum (EcadEC5) (Fannon et al., 1995) was used as a positive reference for the identification of cell–cell adhesion complexes. To obtain a three-dimensional view of the regions of cell–cell contacts, stereo pair images were obtained by tilting the specimen ±5°. Fig. 2 shows a set of stereo pairs of Ep-CAM–stained cells cut through either the horizontal plane (*A*) or through a vertical plane (*B*). As seen in Fig. 2, panels *A*, electron

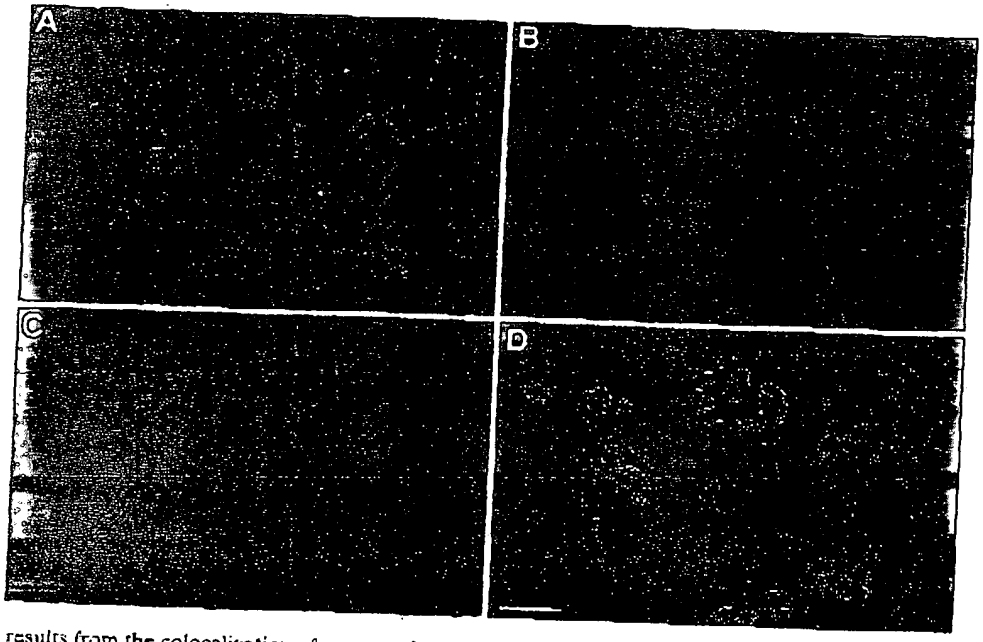

Figure 1. Ep-CAM expression identifies the epithelial compartment of the fetal pancreas. Triple immunofluorescence, followed by confocal microscopic analysis, was performed on cryostat sections from a human fetal pancreas of 18 wk to simultaneously identify Ep-CAM–, insulin-, and glucagon-positive cells. (*A*) Ep-CAM–specific immunoreactivity that highlights the cell boundaries of epithelial cells. The strongest immunoreactivity for Ep-CAM is detected in cell clusters budding from the ducts (*arrowheads*). Notably, many clusters emerging from the ductal epithelium contain large numbers of glucagon- (*B*) and insulin-positive cells (*C*). (*D*) Combined fluorescence spectra. Yellow color results from the colocalization of green and red fluorescences, specific for Ep-CAM and insulin, respectively. Representative of nine independent experiments using five independent donors (18–20 wk of gestation). Bar, 50 μm.

dense stain is specifically localized to regions of cell–cell contacts, delineating the rim of three cells in contact with one another. Stereoviewing of these semithick preparations reveals that staining is discontinuous and concentrated in discrete regions of tight cell–cell association.

Fig. 2, panels *B* represent a stereo pair image collected from an Ep-CAM–stained sample sectioned perpendicularly to the monolayer plan. Viewing from this perspective facilitates the identification of basal, lateral, and apical domains of cells in contact. Notably, most of the electron-dense staining appears targeted to the lateral domain of two cells in contact (*arrow*) and localizes to interdigitated filopodia-like structures contributed to this junction by both cells. Occasionally, some staining localizes to small plasma membrane ruffles at the apical pole of the cells. In contrast, no detectable staining was found on the basal pole of HFP cells, excluding the contribution of Ep-CAM cell interactions with basal membranes.

The high concentration of electron-dense staining observed for Ep-CAM is reminiscent of more typical cell adhesion complexes such as those observed in samples immunostained for E-cadherin (Fig. 3 *A*), a classical epithelial cell adhesion molecule. Fig. 3, panels *B* is a stereo pair of a sample incubated with mouse IgG2a used as negative control for KS1/4 mAb. These data strongly support the involvement of Ep-CAM in the establishment and/or maintenance of epithelial cell–cell interactions.

Ep-CAM Mediates Cell–Cell Adhesion of HFP Epithelial Cells

To investigate the involvement of Ep-CAM in pancreatic epithelial cell–cell adhesion, we performed reaggregation experiments following classical protocols previously used for the functional characterization of other cell adhesion molecules, either in transfected or embryonic cells (Takei-

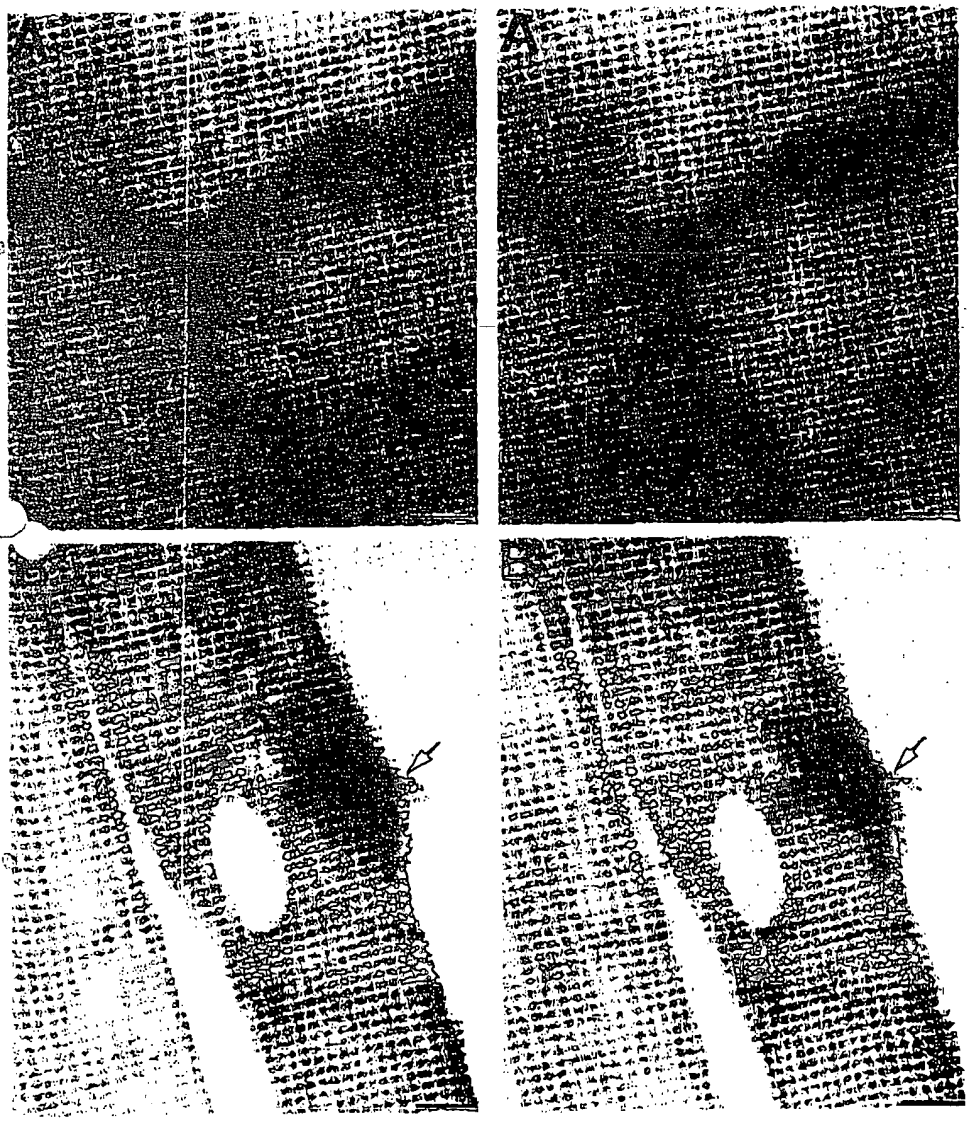

Figure 2. Electron microscopic identification of Ep-CAM at sites of cell–cell contact. Cell monolayers prepared from HFPs were immunolabeled for Ep-CAM by indirect peroxidase method, sectioned either through the horizontal plane (*A*) or a vertical plane perpendicular to the cell monolayer (*B*), and then imaged at 300–400 keV using an intermediate voltage electron microscope (JEOL 4000EX). Stereo pair images were obtained by tilting the specimens +5°. As seen in panels *A*, electron-dense staining specifically localizes to regions of cell–cell contacts, depicting the rim of three cells contacting each other. Panels *B* reproduce a stereo pair image collected from an Ep-CAM-stained sample sectioned through the z axis. This view identifies basal (left end side), lateral, and apical domains (right end side) of cells in contact. Most of the electron-dense staining appears targeted to the lateral domain of two cells in contact (*arrow*), and then localizes to interdigitated philopodia-like structures contributed by both cells. Bars, 1 μm.

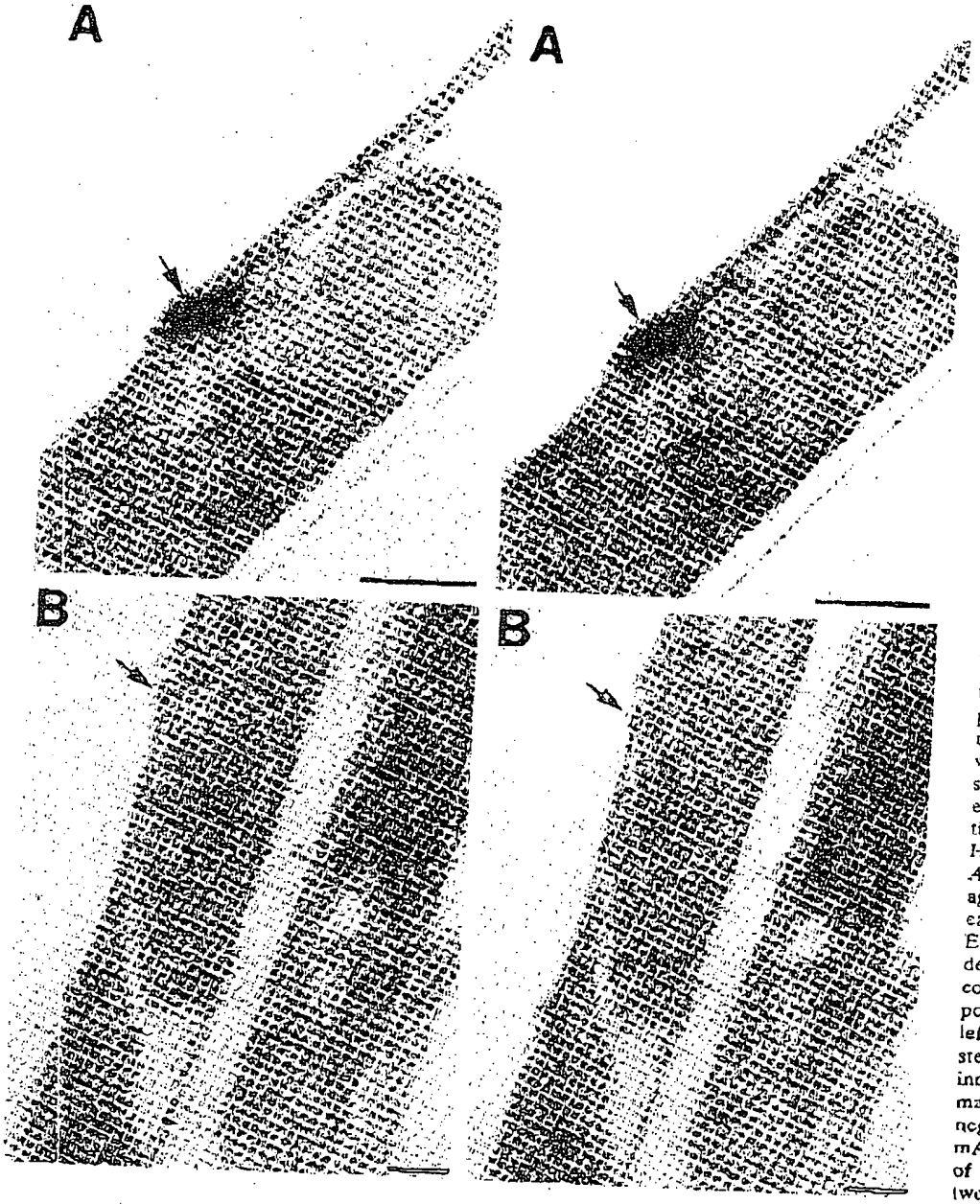

Figure 3. Identification of E-cadherin in junctional complexes of fetal pancreatic epithelial cells. Samples sectioned through a vertical plane and prepared as in Fig. 2 were immunolabeled for E-cadherin with an anti–E-cadherin antiserum (EcadEC5) (Fannon et al., 1995) to identify functional adhesion complexes in HFP cell monolayers. Panels *A* represent a stereo pair image of two cells in contact with each other, displaying strong E-cadherin–specific electron-dense staining at the site of contact (*arrow*). The apical pole of these cells is on the left. Panels *B* reproduce a stereo pair view of a sample incubated with an isotype-matched antibody used as negative control for KS1/4 mAb. The arrow shows a site of cell–cell contact between two cells. Bars, 1 μm.

chi, 1977; Hoffman, 1992), or in primary pancreatic islet cells (Rouiller et al., 1990, 1991; Cirulli et al., 1993). After collagenase digestion of HFPs, small clusters of cells were further dissociated using a nonenzymatic dissociation medium (Sigma Chemical Co.) to obtain a free cell suspension. Immunostaining for Ep-CAM followed by flow cytometric analysis demonstrated that >95% of the cells obtained with this procedure are epithelial. This cell suspension was then used to perform short-term (45 min) reaggregation experiments at 37°C in the presence or absence of calcium, to discriminate between calcium-dependent and calcium-independent adhesion mechanisms (Sperry, 1963). Each of these experimental conditions was also carried out in the presence or absence of anti–Ep-CAM antibodies used either as whole immunoglobulins (mAb 323A3 [Edwards et al., 1986]) or as Fab′ fractions (mAb KS1/4). Fig. 4 shows a representative example of an aggregation assay using HFP cells. As seen in Fig. 4 *A*, incubation of HFP cells for 45 min at 37°C in the presence of calcium and control Fab′ fragments yielded large aggregates that accounted for 74.6% of total aggregation (Fig. 4 *C*, *gray bar*), whereas aggregation performed in the absence of calcium (0.5 mM EDTA) allowed the formation of much smaller cell clusters (Fig. 4 *D*), accounting for only 47% of total aggregation (Fig. 4 *F*, *gray bar*). The qualitative and quantitative difference observed in ion dependence of HFP cell aggregation properties suggest that these cells use both classical calcium-dependent and calcium-inde-

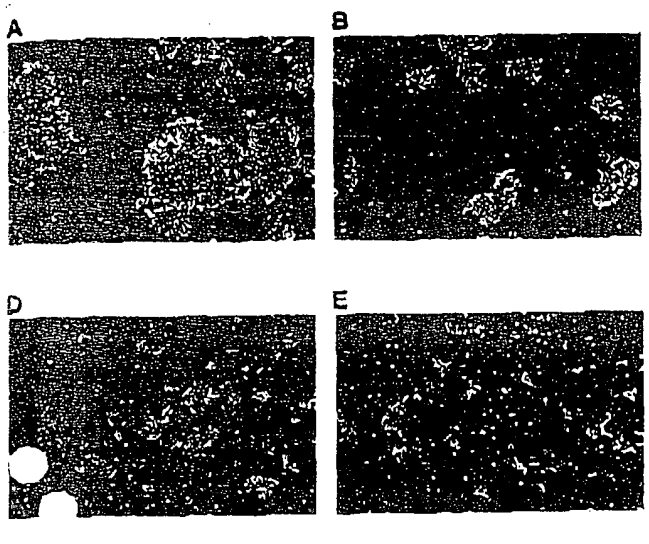

Figure 4. Ep-CAM mediates calcium-independent aggregation of fetal pancreatic epithelial cells. Freshly dissociated pancreatic epithelial cells were reaggregated in the presence (*A–C*) or absence (*D–F*) of calcium, to discriminate between calcium-dependent and -independent adhesion mechanisms. *A* shows the qualitative appearance of cell aggregates obtained in the presence of control Fab' fragments, whereas *B* depicts clusters formed in the presence of anti–Ep-CAM Fab' fragments (from KS1/4 mAb). This mAb reduced the size of cell aggregates, causing an inhibition of cell aggregation from 74.6 to 44% (*C*) ($P < 0.001$). In the absence of calcium, the aggregation observed in the presence of a control Fab' (*D* and *F*) is decreased from 47 to 18.9% (*E* and *F*) ($P < 0.001$). Values in *C* and *F* are expressed as mean ± SEM of four independent experiments, using four independent donors (18–20 wk of gestation).

pendent mechanisms of adhesion to associate to each other. When HFP cells were reaggregated under the same conditions but in the presence of anti–Ep-CAM Fab' fragments (from KS1/4 mAb), reaggregation dynamics were affected both in the presence and absence of calcium. Fig. 4 *B* shows the qualitative appearance of cell clusters obtained in the presence of calcium and anti–Ep-CAM Fab' fragments. Under these conditions, cell aggregation was partially inhibited, yielding much smaller clusters and accounting for only ~44% of total aggregation (Fig. 4 *C*, *open bar*). This residual aggregation is likely to be mediated by cell adhesion molecules of the cadherin superfamily. In contrast, aggregation of HFP cells in the absence of calcium appeared to be dramatically affected by the blockade of Ep-CAM (Fig. 4 *E*). In this condition, very small cell clusters were observed, accounting for only 18.9% of total aggregation (Fig. 4 *F*, *open bar*). Similar inhibitory effects were observed in separate experiments by using mAb 323A3, previously characterized by Litvinov and colleagues as function blocking for Ep-CAM (Litvinov et al., 1984*a*). Our results demonstrate that KS1/4 mAb is also a function-blocking antibody for Ep-CAM, and that Ep-CAM is a major adhesion molecule mediating epithelial cell–cell association in the developing human pancreas.

Expression of Ep-CAM Is Upregulated in Developing Islet Cells Budding from the Ductal Epithelium

Our immunofluorescence studies on HFP at 18 wk of gestation demonstrated a unique expression pattern of Ep-CAM. Fig. 5 shows a representative field collected from a section of HFP stained by two-color immunofluorescence for Ep-CAM (*A*, *green*) and insulin (*B*, *red*). The two fluorophore spectra are merged in Fig. 5 *C*. Although Ep-CAM–specific fluorescence weakly marked ductal epithelial cells (Fig. 5 *C*, *arrow*), it strongly highlighted regions of cell–cell contact within large clusters of epithelial cells budding from the ducts (Fig. 5, *A* and *C*). Some of these cell clusters comprise insulin-producing cells in their core, suggesting that they may represent developing islets of Langerhans. Other epithelial cell clusters, also showing high levels of Ep-CAM, lacked detectable expression of islet hormones. The possibility that these clusters of undifferentiated epithelial cells comprise exocrine precursors remains speculative because no exocrine markers are expressed in the human pancreas at this developmental stage (Fukayama et al., 1986; Jaffe, 1991; Carrere et al., 1992; Mally, 1994, and data not shown). Thus, a unique expression pattern of Ep-CAM characterizes morphologically distinct cell populations in situ, i.e., ductal cells displaying low levels of the antigen, and ICCs and clusters of undifferentiated epithelial cells showing high levels of Ep-CAM. The existence of at least two distinct cell populations identified by Ep-CAM was further confirmed by flow cytometric analysis of cell suspensions. As shown in Fig. 5 *D*, two cell populations are identified by immunostaining with the KS1/4 mAb. One population exhibits low levels of Ep-CAM and accounts for ~30% of total cells (Fig. 5 *D*, *arrow*), whereas the other displays high levels of Ep-CAM expression and accounts for ~65% of total cells (Fig. 5 *D*, *arrowhead*). We suggest that Ep-CAM^{low} cells identified by flow cytometry are likely to correspond in situ to ductal cells (Fig. 5 *C*, *arrow*), whereas the Ep-CAM^{high} cell population (Fig. 5 *D*, *arrowhead*) comprises developing ICCs (Fig. 5 *C*, *arrowheads*) and undifferentiated epithelial cells. Overall, the data show that modulation of Ep-CAM expression accompanies the budding of islet cells from the ductal epithelium.

Expression Pattern of Ep-CAM in the Human Adult Pancreas

To investigate whether the Ep-CAM expression pattern observed in fetal pancreata is retained in the adult pancreas, we performed triple immunofluorescence experiments for the simultaneous identification of Ep-CAM, insulin, and glucagon in cryostat sections from adult human pancreas. In contrast to fetal pancreas, the highest levels of expression of Ep-CAM were not identified in endocrine cells, but rather in small intercalar ducts, interlobular, and

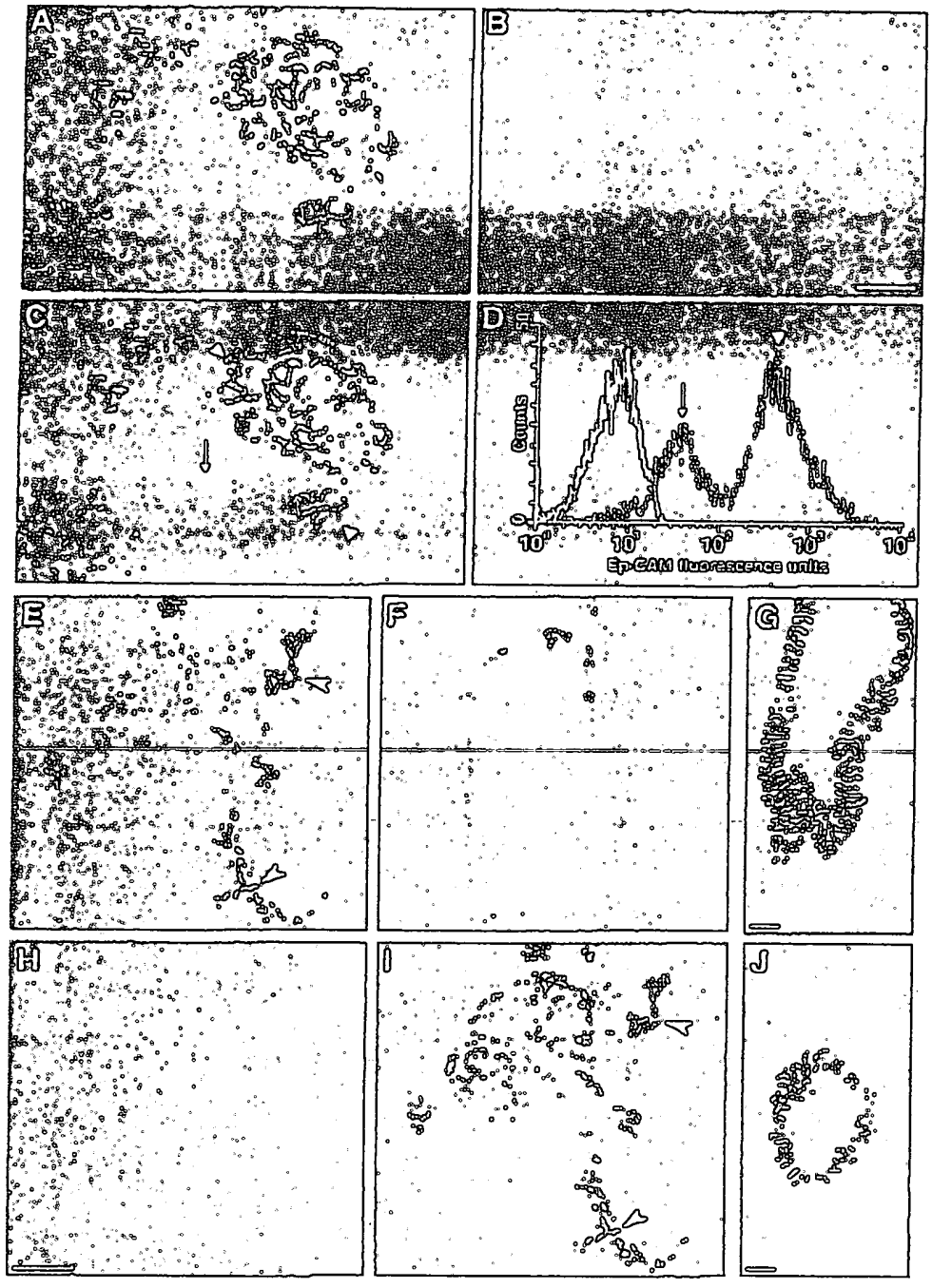

Figure 5. Developmentally regulated expression of Ep-CAM in the human pancreas. Confocal analysis performed on cryostat sections of a HFP, double immunostained for Ep-CAM (*A*) and insulin (*B*), reveals the upregulation of Ep-CAM expression in cell clusters budding from the ductal epithelium (*A*). The two fluorophore spectra are merged in *C*, where insulin-positive cells (*red*) are identified within clusters displaying the brightest immunoreactivity for Ep-CAM (*green*). *D* represents a typical histogram obtained by flow cytometric analysis after immunolabeling for Ep-CAM of freshly isolated HFP cells. It shows that two distinct populations of Ep-CAM–positive cells are present in the HFP, an Ep-CAM^{low} (*arrow*) and an Ep-CAM^{high} (*arrowhead*) population. This pattern, when compared to the in situ detection of Ep-CAM (*A* and *C*), suggests that Ep-CAM^{low} cells comprise ductal cells (*C* and *D*, *arrows*), whereas Ep-CAM^{high} cells (*C* and *D*, *arrowheads*) correspond to developing islet cells, and clusters of undifferentiated epithelial cells. The white tracing in *D* represents the autofluorescence of cells incubated with a mouse IgG2a used as a control reference. The data are representative of nine independent immunostainings using five independent donors (18–20 wk of gestation). Triple immunofluorescent localization was also performed on sections of human adult pancreas to simultaneously identify Ep-CAM, insulin, and glucagon. In this set of experiments, the brightest Ep-CAM–specific immunofluorescence was recorded at the cell–cell boundaries of intercalar ductal cells (*E*, *arrowheads*), in interlobular ducts (*J*), and in main ducts (*G*). Islets of Langerhans, identified by the insulin- (*H*) and glucagon-specific fluorescence (*F*), exhibit a significantly less intense Ep-CAM–specific fluorescence (*E*). Combined fluorophore spectra (*I*). The data are representative of four independent experiments, using three independent adult donors (20–56 yr old). Bars: (*B*) 30 μm; (*H* and *G*) 50 μm; (*J*) 40 μm.

main ducts. Indeed, as shown in Fig. 5, *E* and *I*, adult islets, identified by the insulin (Fig. 5 *H*) and glucagon staining (Fig. 5 *F*), displayed low to intermediate levels of Ep-CAM as compared to the bright fluorescent signal recorded at the cell–cell boundaries of intercalar ductal cells (Fig. 5, *E* and *I*, *arrowheads*), and in interlobular and main ducts (Fig. 5, *G* and *J*). Often localized intracellularly, exocrine tissue demonstrated weak staining for Ep-CAM.

Table I. Pixel Intensity of Ep-CAM–specific Immunofluorescence at Regions of Cell–Cell Contact

cell types	Fetal pancreas	Adult pancreas	ICCs	Grafts
ductal	73.2 ± 12.6*	179.4 ± 18.3‡	71.1 ± 11.7§	177.9 ± 21.3¶
endocrine	180.3 ± 21.5	110.8 ± 11.2	185.4 ± 13.5	104.8 ± 17.6
epithelial undifferentiated	197.9 ± 22.5	–	194.1 ± 17.2	–
exocrine	–	56.8 ± 7.1	–	–

The pixel intensity of Ep-CAM–specific fluorescence was recorded in 250 domains of cell–cell contact for each cell type. Numbers of fields scored in fetal pancreata = 50, adult pancreata = 72, ICCs = 54, grafts = 69. Number of donors = 3 (fetal pancreata at gestational age 18–20 wk). Number of donors of adult pancreata = 3 (age: 20, 32, and 56 yr).
The pixel intensity recorded in ductal cells is significantly different from values detected in endocrine cells and epithelial undifferentiated cells ($P < 0.001$).
‡ Significantly different from values detected in endocrine cells ($P < 0.05$) and in exocrine cells ($P < 0.001$).
§ Significantly different from values detected in endocrine and epithelial undifferentiated cells ($P < 0.001$).
‖ Values recorded in grafts' ductal cells differ significantly from those recorded in endocrine cells ($P < 0.05$).

Quantitative Analysis of Ep-CAM Expression in Fetal and Adult Human Pancreas

To quantitatively evaluate the levels of Ep-CAM expression in specific cell types, confocal images from human fetal and adult pancreata were studied by computer-aided morphometric analysis. Table I shows the pixel intensities of Ep-CAM–specific fluorescence recorded in ductal, endocrine, and epithelial undifferentiated cells of the fetal pancreas, as well as in ductal, islet, and acinar cells of the adult pancreas. Whereas fetal ductal cells displayed a mean pixel intensity of 73.2 ± 12.6, islet cells within the same sections showed a mean pixel intensity of 180.3 ± 21.5 ($P < 0.001$). Similarly, high levels of Ep-CAM expression (i.e., 197.9 ± 22.5) were measured in clusters of epithelial undifferentiated cells that lack expression of all islet hormones (insulin, glucagon, pancreatic polypeptide, and somatostatin), or exocrine markers such as amylase (data not shown). Conversely, this analysis performed on adult pancreas revealed mean pixel intensities of 179.4 ± 18.3 in ductal cells and 110.8 ± 11.2 in islet cells ($P <$ 0.05). Cells of the exocrine compartment showed significantly lower pixel intensity (56.8 ± 7.1). Thus, ductal and islet cells demonstrate a reverse pattern of Ep-CAM expression in the adult pancreas compared to the fetal pancreas. These results suggest that mechanisms regulating high levels of Ep-CAM expression in fetal islets and undifferentiated cell clusters may remain constitutively operative in adult ductal cells, perhaps marking the ability of this cell compartment to act as a reservoir of islet cell progenitors in adult life (Dudek et al., 1991; Gu et al., 1993).

High Levels of Ep-CAM Expression Characterize Proliferating Epithelial Cells In Situ

The high levels of Ep-CAM expressed by developing islet cell clusters in the fetal pancreas suggest that upregulation of Ep-CAM may be associated with cell proliferation. To test this hypothesis, fetal and adult human pancreata were stained for Ep-CAM, insulin, and the Ki-67 antigen, a nuclear marker of cycling cells (Gerdes et al., 1983). The presence of proliferating events among ductal, endocrine, and exocrine cells was evaluated both qualitatively (Fig. 6) and quantitatively by morphometric analysis of serial tissue sections (Table II). Fig. 6 *A* represents a reconstruction of four adjacent fields from a fetal pancreas showing a large duct (*asterisks*) and several adjacent cell clusters. In no instances were Ki-67-positive nuclei (Fig. 6 *A*, *blue*) identified among ductal cells weakly stained for Ep-CAM. Conversely, proliferating ductal cells consistently exhibited strong staining for Ep-CAM (Fig. 6 *A*, *arrowheads*). Among cells adjacent to the ducts, numerous Ki-67–positive Ep-CAM^{high} cells could also be observed. Nevertheless, many Ep-CAM^{high} cells appeared to be not cycling. Within cell clusters budding from the ductal epithelium, several insulin-producing cells (Fig. 6, *arrows*) also positive for the Ki-67 antigen were observed. This latter observation suggests that newly differentiated β cells may contribute to the expansion of the islet cell mass. The fact that developing islet cells exhibited high levels of Ep-CAM is consistent with an immature phenotype, as supported by their inability to secrete hormones in response to secretagogues.

Similar analysis performed on cryostat sections from human adult pancreata revealed fewer cycling events (Fig. 6, *B–D*). Fig. 6 *B* shows an islet of Langerhans identified by the insulin-specific staining (*red*). Relatively higher levels of Ep-CAM expression (Fig. 6, *green*) were detected in islet cells as compared to the surrounding acinar tissue. In this field, a cycling insulin-positive cell can be identified by the expression of Ki-67 (Fig. 6, *blue*) in its nucleus (Fig. 6, *arrow*). Fig. 6 *C* shows Ki-67–positive nuclei within the ductal epithelium (*arrows*), a cell compartment that expresses the highest levels of Ep-CAM in the adult pancreas. Interestingly, even within the exocrine compartment, which overall expresses low levels of Ep-CAM, cycling cells identified by the Ki-67 staining always showed brighter Ep-CAM–specific fluorescence, as compared to the surrounding noncycling acinar cells (Fig. 6 *D*, *arrows*). Table II summarizes the frequency of Ki-67–positive nuclei in the different cell types of both the fetal and adult human pancreas. To take into account the different representation of the various pancreatic cell types, the percentage of Ki-67–positive cells was corrected for the relative cell mass of the ductal, endocrine, and exocrine pancreatic compartments. This allowed the calculation of a "proliferation index" that reflected the actual frequency of proliferating cells in each compartment. This analysis demonstrates that the cells bearing high levels of Ep-CAM expression (i.e., undifferentiated epithelium in the fetal pancreas and ductal cells in the adult pancreas) exhibit a higher proliferation index (Table II), consistent with the hypothesis that upregulation of Ep-CAM is linked to the high proliferative potential of those cell compartments.

Induction of Epithelial Cell Growth Is Associated with the Upregulation of Ep-CAM Expression

To determine whether upregulation of Ep-CAM is associ-

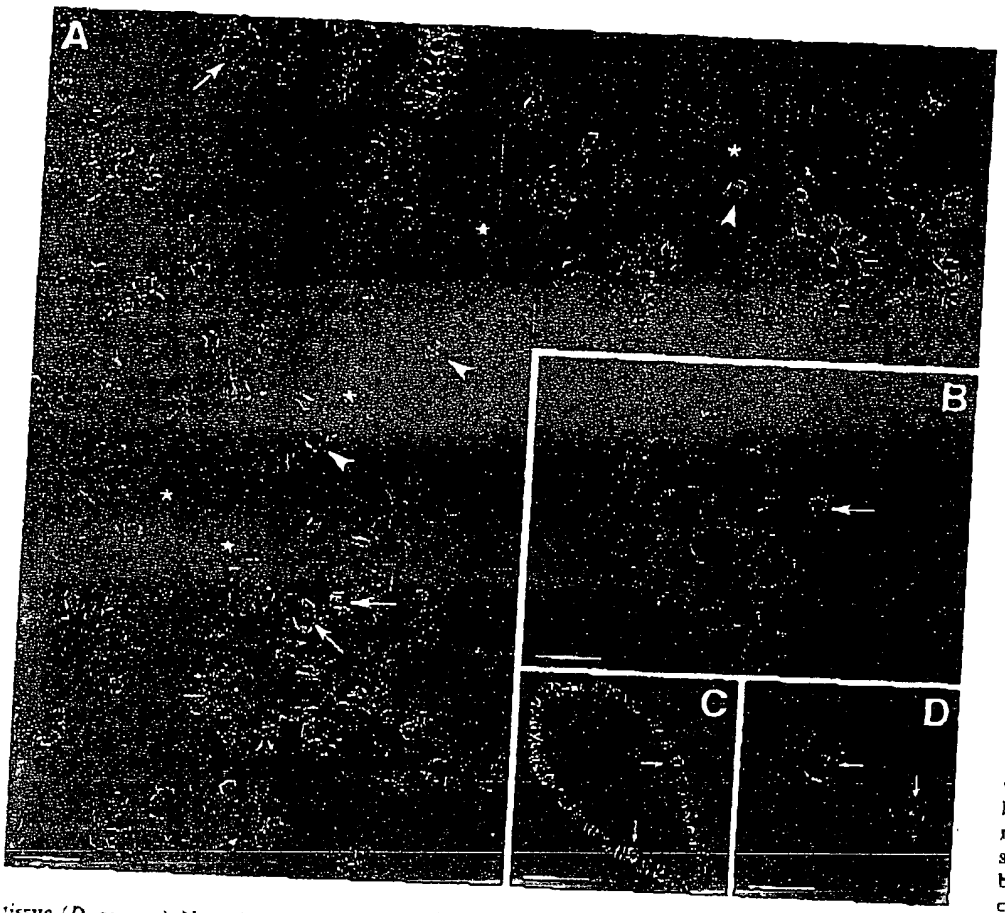

Figure 6. High levels of Ep-CAM expression mark proliferating pancreatic epithelial cells in situ. *A* shows a reconstruction of four adjacent microscopic fields from a fetal pancreas (18 wk), immunostained for Ep-CAM (*green*), insulin (*red*), and Ki-67 (*blue*). Numerous proliferating cells identified by the nuclear staining for Ki-67 can be observed in cell clusters budding from pancreatic ducts (*asterisks*), and within the ductal epithelium (*arrowheads*). Notably, all Ki-67–positive cells exhibited high levels of Ep-CAM–specific immunoreactivity, including those identified within the monolayered ductal epithelium that normally exhibit low levels of Ep-CAM. Many cycling insulin-positive cells were also observed (*arrows*). Similar analysis performed on sections of human adult pancreas (*B–D*) also revealed that expression of Ki-67 is always associated with high levels of Ep-CAM. Although rare cycling β cells were observed (*B, arrow*; refer to Table II), most Ki-67–positive cells were identified in ducts (*C, arrows*) and in the acinar tissue (*D, arrows*). Note that even within the exocrine compartment, which overall expresses low levels of Ep-CAM, cycling cells identified by the Ki-67 staining always showed brighter Ep-CAM–specific fluorescence compared to the surrounding noncycling acinar cells. The data are representative of five independent experiments, using three independent donors of both fetal (18–20 wk of gestation) and adult pancreata (20–56 yr old). Bars: (*A*) 50 μm; (*B* and *C*) 80 μm; (*D*) 40 μm.

ated with the growth of pancreatic epithelial cells in vitro, we next assessed the expression levels of this antigen by protein immunoblotting. We compared free-floating ICCs, comprising only a limited number of cycling cells (Otonkoski et al., 1993), to cell monolayers generated by culturing fetal pancreatic cells on the 804G extracellular matrix supplemented with rhHGF/SF. This culture condition greatly enhances the proliferation of both fetal (Beattie et al., 1996) and adult human islet cells (Hayek et al., 1995). As shown in Fig. 7, lane *1*, a major band with an apparent molecular mass of 40 kD could be identified by the KS1/4 mAb in cell extracts from fetal ICCs cultured as floating clusters. Notably, when cells were plated on the 804G extracellular matrix to promote epithelial cell growth, a remarkable upregulation of Ep-CAM expression was observed (Fig. 7, lane *2*). In this latter condition, an additional 42-kD band was also detected. The 40- and 42-kD proteins recognized by the KS1/4 mAb were previously characterized in UCLA-P3 cells as differently glycosylated isoforms of the same antigen (Perez and Walker, 1989). Induction of cell growth in adult islets cultured as monolayers was also associated with a significant upregulation of Ep-CAM (Fig. 7, lane *5*) as compared to the levels detected in floating islet cultures (Fig. 7, lane *4*). Interestingly, lysates from a human islet β cell tumor (insulinoma) exhibited higher levels of Ep-CAM expression than normal islets (Fig. 7, lanes *8* and *7*, respectively). These results indicate that islet cell growth induced by mitogenic stimula in vitro or tumoral transformation in vivo is accompanied by increased expression of Ep-CAM.

Developmentally Regulated Expression of Ep-CAM during Pancreatic Islet Maturation

To follow the expression of Ep-CAM during development of islet clusters, we took advantage of an established in vivo model of endocrine differentiation of HFP cells. In this model, undifferentiated fetal pancreatic ICCs transplanted under the kidney capsule of immune-deficient nude mice develop a significant endocrine cell mass after a 12-wk period (Beattie et al., 1995, 1996; Hayek et al., 1996). Therefore, in the next series of experiments we transplanted four nude mice with 500 human ICCs each. After 12 wk, the mice were tested for the presence of human insulin in the peripheral blood using a human C-peptide–specific radioimmunoassay. We detected 81 ± 20 pmol/li-

Table II. Distribution of Ki-67–positive Cells in the Human Pancreas

Cell types	Ki-67–positive cells	Proliferation index
Fetal pancreas		
Ductal	11.70 [122]*	0.48‡
Endocrine	14.87 [155]	0.69
Epithelial undifferentiated	52.01 [542]	1.50
Adult pancreas		
Ductal	39.12 [367]§	2.79‖
Endocrine	0.95 [9]	0.47
Acinar	44.80 [421]	0.60

*Expressed as percentage of total number of cells counted. [number of cells counted].
‡Calculated by correcting the percentage of Ki-67–positive cells for the relative proportion of pancreatic cell types (i.e., ductal cells = 24%, endocrine cells = 21.5%, epithelial undifferentiated = 34.5%, stroma = 20%) (Cirulli, V., unpublished data). Number of fields scored = 96. Total number of cells counted = 819. Number of donors = 3 (fetal pancreata at gestational age 18–20 wk).
§Expressed as percentage of total number of cells counted. [Number of cells counted].
‖Calculated by correcting the percentage of Ki-67–positive cells for the relative proportion of the different pancreatic cell types (i.e., ductal cells = 14%, endocrine cells = 2[illegible] epithelial undifferentiated = 74%, stroma = 10%) (for review see Githens, 1983). Number of fields scored = 202. Total number of cells counted = 797. Number of donors = 3 (age: 20, 32, and 56 yr).

ter of human C peptide in basal conditions and 424.5 ± 27.8 pmol/liter after intraperitoneal injection of glucose, indicating that the transplanted mice harbored functionally mature human islet β cells. Hematoxylin/eosin staining of the grafts showed numerous large islet-like epithelial clusters (Fig. 8 *B*, *arrowheads*) and ductal structures (Fig. 8 *B*, *asterisks* and *inset*). Consistent with our previous reports, such islet cell clusters were immersed in a well-vascularized stromal tissue of mouse origin (Beattie et al., 1994, 1997). None of the grafts developed exocrine tissue, as detected by morphological analysis and immunostaining for amylase (data not shown). This last observation is in agreement with a recent report by Gittes and colleagues, showing that transplantation of enriched epithelial cell preparations from the mouse fetal pancreas develops into islet cells and ductal elements without exocrine tissue (Gittes et al., 1997).

We next compared the expression of Ep-CAM in the grafts and in samples of ICCs preserved for histological analysis from the very same pool used to transplant the nude mice (Fig. 8 *A*). For this purpose, sections from the ICC preparations and grafts were stained by three-color immunofluorescence for Ep-CAM (*green*), insulin (*red*), and glucagon (*blue*). The ICC preparations (Fig. 8, *C* and *D*) consisted mostly of epithelial cells strongly stained for Ep-CAM (pixel intensities were 194.1 ± 17.2 in epithelial undifferentiated cells and 185.4 ± 13.5 in endocrine cells, respectively; refer to Table I). Few ductal structures (Fig. 8 *C*, *arrows*) exhibiting low levels of Ep-CAM–specific staining (pixel intensity of 71.1 ± 11.7; refer to Table I), and discrete regions occupied by mesenchymal cells lacking Ep-CAM expression (Fig. 8 *C*, *arrowhead*) were also observed. Merging of the three fluorophore spectra in Fig. 8 *D* showed that only a minor fraction of the epithelial cells express insulin and/or glucagon, consistent with the fact that ICCs comprise mostly undifferentiated epithelial cells (Beattie et al., 1997). In contrast, the analysis of the grafts revealed the presence of large clusters of endocrine cells resembling adult islets of Langerhans (Fig. 8, *E–H*), with insulin-producing cells located in the center and most glucagon cells arranged at the periphery. In addition, well-formed ducts containing numerous glucagon-producing cells were observed (Fig. 8 *H*, *arrows*), suggesting an ongoing and/or residual emergence of endocrine cells from the ductal epithelium. Pancreatic polypeptide- and somatostatin-positive cells were also found in the grafts (data not shown). Interestingly, the expression pattern of Ep-CAM in these grafts is reminiscent of that observed in the intact adult human pancreas, with high levels in the ductal epithelium and relatively lower levels in islet cells (Fig. 8 *E*; pixel intensity of 104.8 ± 17.6 in islet cells versus 177.9 ± 21.3 in ductal cells, respectively; refer to Table I). These results indicate that differentiation of fetal cells into functional mature endocrine cells is associated with downregulation of Ep-CAM.

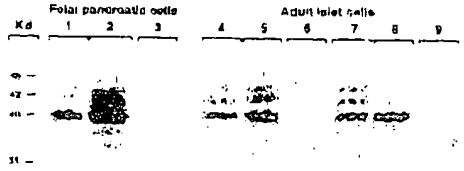

Figure 7. Ep-CAM expression is upregulated by growth stimuli. Western blot analysis of cell extracts from fetal ICCs (lane *1*) revealed a band with an apparent molecular mass of 40 kD using the KS1/4 mAb. When cells were plated on the 804G extracellular matrix to promote epithelial cell growth, a remarkable upregulation of Ep-CAM expression was observed (lane *2*). In this condition, an additional band of 42 kD was detected. When purified adult islets were cultured either in the absence of growth stimuli (lane *4*), or as monolayers on the 804G matrix supplemented with rhHGF/SF (lane *5*), a significant upregulation of Ep-CAM expression was observed. Lysates from human insulinomas also showed higher levels of Ep-CAM (lane *8*) compared to normal islets (lane *7*). Lanes *3*, *6*, and *9* were loaded with cell lysates from fetal cell monolayers, adult islet monolayers, and human insulinoma, respectively, and then probed with a control mouse IgG2a. The data are representative of seven independent experiments using fetal and adult cells (seven and five independent donors, respectively), and of three independent experiments using human insulinomas (from two independent donors).

Inhibition of Ep-CAM–mediated Cell–Cell Interactions Causes Endocrine Differentiation of HFP Cells

The expression pattern of Ep-CAM in the developing human pancreas suggests that a dynamic modulation of this CAM may be involved in the cell's decision to either grow or differentiate. To assess whether the blockade of Ep-CAM–mediated cell–cell interactions may mimic a state of functional downregulation of Ep-CAM usage/expression and promote endocrine differentiation, HFP cells were cultured as floating ICCs for 5 d in the presence of either Fab′ fragments of KS1/4 mAb or control Fab′ generated from isotype-matched mouse IgGs. At the end of the culture period, control and KS1/4-treated cells were harvested for total RNA isolation and DNA and insulin content determination. Because human HFP express a number of other adhesion molecules, including N-CAM and E-cadherin (Cirulli et al., 1995), we predicted that treatment with KS1/4 Fab′, although exerting a potent inhibitory effect on short-term reaggregation experiments (refer to Fig. 4), should have not affected their ability to reaggregate in

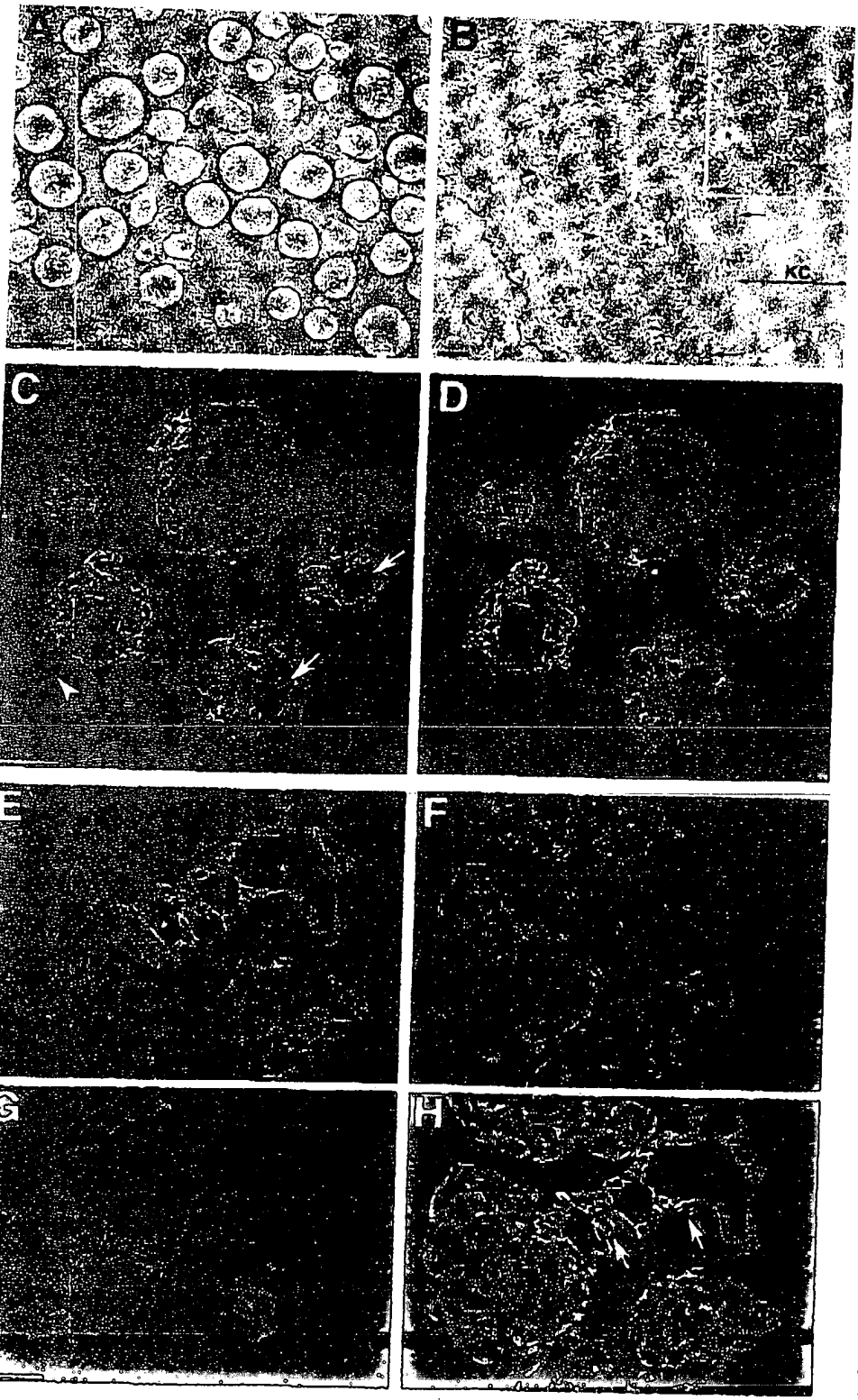

Figure 8. Developmentally regulated expression of Ep-CAM during pancreatic islet maturation. Fetal pancreatic cells cultured as floating ICCs (*A*) were transplanted under the kidney capsule of nude mice, and grafts were analyzed after 12 wk for the presence of islet tissue (*B*). Hematoxylin-eosin staining of graft sections revealed the presence of numerous ICCs (*arrowheads*) and some ductal elements (*asterisks*) immersed in a well-vascularized stroma (*arrows* indicate blood vessels). *K*, mouse kidney; *KC*, kidney capsule. Three-color immunofluorescence for the simultaneous identification of Ep-CAM (*green*), insulin (*red*), and glucagon (*blue*), was performed in sections from ICCs. *C* shows the Ep-CAM–specific immunofluorescence that highlights cell–cell boundaries of epithelial cells. Few ductal structures can be identified within these cell clusters (*arrows*), displaying a dimmer Ep-CAM–specific signal, as in fetal pancreas in situ. A small fraction of mesenchymal cells (<10%) lacking Ep-CAM expression can be identified in some ICCs (*arrowheads*). Merging of the three fluorophore spectra (*D*), specific for Ep-CAM (*green*), insulin (*red*), and glucagon (*blue*), shows that only a small fraction of the cells forming ICCs express islet hormones (5–10%), whereas most of the other epithelial cells are undifferentiated. Immunostaining of sections from the grafts after 12 wk in vivo shows that high levels of Ep-CAM (*E*) are found in ductal elements (*asterisks*), whereas endocrine cells (*glucagon*, *F*; *insulin*, *G*) exhibit lower levels, as in the adult pancreas in situ. *H* shows the merging of the three fluorophore spectra specific for Ep-CAM (*green*), insulin (*red*), and glucagon (*blue*). Bars: (*A*) 100 μm; (*B*, *C*, and *G*) 50 μm.

long-term cultures into ICCs. In fact, after 5 d of culture both control and KS1/4-treated samples yielded macroscopically similar ICCs (data not shown). As shown in Fig. 9 *A*, analysis of insulin and glucagon transcripts revealed that treatment with anti–Ep-CAM KS1/4 Fab' causes a significant increase of both insulin and glucagon gene transcription. Nicotinamide-treated ICCs (Fig. 9, lane *NIC*) were used in this assay as an internal positive control for induction of endocrine differentiation. In fact, we have previously shown that nicotinamide increases glucagon and insulin gene transcription, as well as the overall number of glucagon- and glucose-responsive insulin-producing cells (Otonkoski et al., 1993). Quantitation of band intensities performed by scanning densitometry showed that the blockade of Ep-CAM (Fig. 9, lane *KS1/4 Fab'*) produced a 1.9- and 1.4-fold increase, respectively, of insulin and glucagon mRNAs above the levels measured in control ICCs (Fig. 9 *B*). These findings were further validated by the demonstration of increased insulin protein content in ICCs treated with anti–Ep-CAM KS1/4 Fab' (Fig. 9 *C*).

These results strongly suggest that disengagement of Ep-CAM–mediated cell–cell interactions, by either downregulation of the receptor from the cell surface and/or functional inactivation, may be a signaling event involved in the differentiation of pancreatic islet cells.

Discussion

Adhesion molecules mediating cell–cell interactions play crucial morphoregulatory roles in specifying cell fate during development (Trelstad, 1984; Ekblom et al., 1986; Edelman and Crossin, 1991; Edelman, 1992; Takeichi, 1991, 1995). Here we demonstrate that the pancarcinoma antigen KSA (Varki et al., 1984; Edwards et al., 1986; Spurr et al., 1986; Bumol et al., 1988), alias Ep-CAM (Litvinov et al., 1994*a*), is expressed in the human fetal pancreas at sites of epithelial cell–cell contacts and that the antigen mediates a calcium-independent mechanism of cell aggregation. The data also show that expression of Ep-CAM is developmentally regulated during pancreatic islet ontogeny. Thus, high levels of Ep-CAM expression were detected in fetal pancreatic islet cells in situ and after induction of cell growth in vitro. In contrast, endocrine differentiation of immature fetal pancreatic epithelial cells in an in vivo transplantation model was associated with a significant downregulation of Ep-CAM expression. Moreover, blockade of Ep-CAM function in vitro caused endocrine differentiation of fetal pancreatic cells. Taken together, these observations suggest that Ep-CAM exhibits the features of a morphoregulatory molecule.

Our data provide evidence that Ep-CAM upregulation and function is associated with the outgrowth of early endocrine cells from the pancreatic ductal epithelium. We show that Ep-CAM expression is restricted to the epithelial compartment of the fetal pancreas in a pattern reminiscent of the distribution of classical cell–cell adhesion molecules. Furthermore, immunoelectron microscopic analysis of Ep-CAM showed that the predominant subcellular localization of this glycoprotein is targeted to domains of cell–cell contact, suggesting its involvement in cell aggregation. Indeed, our short-term reaggregation experiments clearly demonstrate that Ep-CAM is involved in pancreatic epithelial cell–cell aggregation. Hence, using two different Ep-CAM–specific mAbs, KS1/4 and 323A3, both recognizing extracellular sequences of the molecule, a significant inhibitory effect of pancreatic epithelial cell reaggregation was observed. These functional data provide the first evidence that Ep-CAM mediates cell–cell aggregation of primary (nontumoral) epithelial cells. Based on some sequence homology that Ep-CAM shares with the extracellular matrix protein nidogen, it was previously suggested that this epithelial marker could participate in either intercellular or cell–matrix adhesion of epithelial cells (Thampoe et al., 1988; Mann et al., 1989; Simon et al., 1990). Although a direct involvement of Ep-CAM in cell–cell interactions is supported by our morphological studies and demonstrated by our antibody perturbation experiments, the possibility of Ep-CAM involvement in cell–matrix interactions is unlikely for the following reasons. First, Ep-CAM–specific immunoreactivity was never detected at the basal pole of pancreatic epithelial cells, as shown by

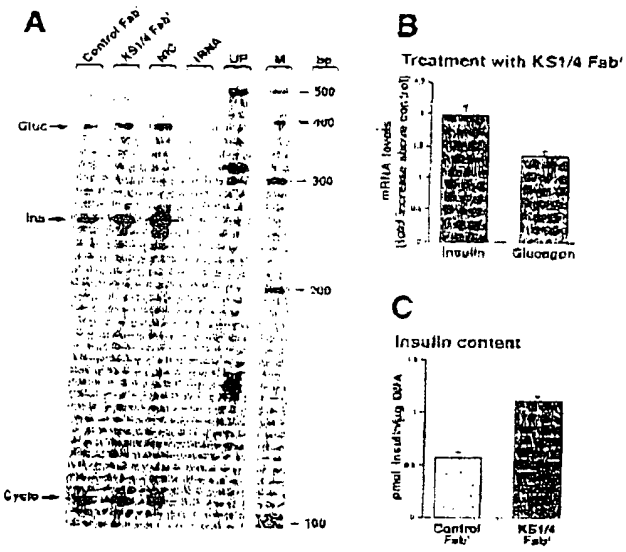

Figure 9. Blockade of Ep-CAM–mediated cell–cell interactions causes endocrine differentiation of HFP cells. HFP cells cultured as floating ICCs for 5 d in the presence of either Fab' fragments of KS1/4 mAb or control Fab' were used for determination of insulin and glucagon transcript levels in a multiprobe RNase protection assay. *A* shows that treatment with anti–Ep-CAM KS1/4 Fab' causes a significant increase of both insulin and glucagon gene transcription. Nicotinamide-treated ICCs (lane *NIC*) were used in the assay as an internal positive control for induction of endocrine differentiation. Yeast tRNA (lane *tRNA*) was included as a negative control. Lane *UP*, undigested probes; *M*, RNA sizing ladder. Quantitation of band intensities performed by scanning densitometry (*B*) shows that blockade of Ep-CAM produces a 1.9- and 1.4-fold increase, respectively, of insulin and glucagon mRNAs above the levels measured in control ICCs. Note that the insulin protein content is also increased in ICCs treated with anti–Ep-CAM KS1/4 Fab' (*C*). *A* is representative of four independent assays. Data in *B* and *C* are expressed as mean ± SEM of four independent experiments. *P* values for significant differences were: $P < 0.002$ for both insulin and glucagon transcript levels compared to control in *B*; $P < 0.005$ for samples treated with KS1/4 Fab' compared to control in *C*.

our electron microscopic analysis, and secondly, we found that both KS1/4 and 323A3 mAbs are unable to inhibit pancreatic cell adhesion to the 804G matrix (data not shown).

We provide evidence that upregulation of Ep-CAM is linked to cell proliferative events. Thus, cycling cells, identified in situ by the expression of the Ki-67 antigen, consistently displayed the highest levels of Ep-CAM expression. In the fetal pancreas, the highest frequency of proliferating cells was found among Ep-CAM^{high} ICCs budding from the ducts. Conversely, in the adult pancreas, the ductal compartment strongly stained for Ep-CAM exhibited the highest proliferation index. Interestingly, in no instances were Ep-CAM^{low} cells found to proliferate. In fact, we observed that cell compartments such as the fetal ductal epithelium and the adult acinar tissue that overall exhibited low levels of Ep-CAM comprised scattered proliferating cells that expressed increased levels of Ep-CAM. Thus, our studies establish a direct correlation between frequency of proliferating cells and high expression of Ep-CAM in each cell compartment. However, we found that not all Ep-CAM^{high} cells within fetal islet cell clusters and adult ducts are actually cycling. This latter observation suggests that upregulation of Ep-CAM may not necessarily coincide with cell division, but rather marks the capability of epithelial cells to respond to an array of mitogenic/developmental signals.

In line with this hypothesis is the observation that remarkable upregulation of Ep-CAM occurs not only in fetal epithelia but also in rapidly growing carcinomas (Varki et al., 1984). Thus, it is possible that cell interactions mediated by this glycoprotein, although ensuring cell–cell association, may be required for the transduction of specific signals necessary for the development of various epithelial organs and for the growth of epithelial tumors. Hence, the association of Ep-CAM upregulation with cell growth in tumors of epithelial origin (Edwards et al., 1986; Moldenhauer et al., 1987; Litvinov et al., 1994*a*,*b*; Velders et al., 1994), and its de novo expression during tumoral transformation in tissues that usually lack this molecule have been extensively documented (Quak et al., 1990; Tsubura et al., 1992; Tellechea et al., 1993). Tumoral transformation of epithelial tissues has been also associated with a decreased expression/function of E-cadherin, a CAM required for the development and maintenance of cellular differentiation (Marrs and Nelson, 1996), and regarded as a tumor suppressor gene (Takeichi, 1993). Interestingly, a recent report by Litvinov and colleagues shows that Ep-CAM can decrease cadherin-mediated cell–cell association, thereby providing a possible mechanism by which Ep-CAM may promote the development of a proliferative phenotype in epithelial cells (Litvinov et al., 1997).

Based on the evidence that pancreatic endocrine cells derive from progenitors resident in the ductal epithelium (Pictet and Rutter, 1972; Pictet et al., 1972; Teitelman and Lee, 1987; Alpert et al., 1988; Gu and Sarvetnick, 1993), our data suggests that upregulation of Ep-CAM occurs at an early developmental stage of islet cell ontogeny. In support of this hypothesis we show that Ep-CAM is upregulated in pancreatic epithelial and islet cells induced to proliferate in vitro, and in spontaneous pancreatic β cell tumors in vivo. We propose that the biological significance of Ep-CAM upregulation in vivo is to transduce specific morphogenetic signals during the emigration and expansion of early endocrine precursors and newly differentiated endocrine cells into the surrounding mesenchyme.

The expression pattern of Ep-CAM that we describe in the fetal and adult pancreas, as well as in our in vivo model of islet cell development from undifferentiated fetal epithelial cells, provides an interesting example of the developmentally regulated expression of this antigen. Thus, whereas in the fetal pancreas the highest levels of Ep-CAM are detected in islet cell clusters growing out of the ductal tree and in clusters of undifferentiated epithelial cells, in adult life the ductal epithelium, rather than islet cells, displays the strongest Ep-CAM–specific immunoreactivity. These observations suggest that human adult pancreatic ducts may retain a higher responsiveness to growth stimuli, a characteristic supported by our data on the relatively higher frequency of proliferative events in this cell compartment. This possibility is also consistent with the current belief that most human adult pancreatic tumors are of ductal origin.

The in vivo and in vitro experimental models of endocrine differentiation described here provide additional evidence for Ep-CAM playing important morphoregulatory roles in pancreatic islet development. Thus, a significant downregulation of Ep-CAM was observed when undifferentiated fetal pancreatic epithelial cells were transplanted and allowed to differentiate in vivo. Hence, upon endocrine differentiation, islet cell clusters developed within the grafts and expressed levels of Ep-CAM similar to those detected in situ in the adult islets. Likewise, when the adhesive function of Ep-CAM was inhibited by specific KS1/4 Fab′ fragments, fetal pancreatic cells underwent endocrine differentiation, demonstrated by the upregulation of insulin and glucagon transcripts, as well as by the increased insulin protein content. These data strongly suggest that manipulations designed to inhibit or reduce Ep-CAM–mediated interactions favor endocrine differentiation. Conversely, upregulation of Ep-CAM negatively regulates endocrine differentiation. In line with this view, the low levels of Ep-CAM expressed in human adult islets may be ineffective in inhibiting hormone gene expression, and only a substantial upregulation of this CAM above threshold levels can endow epithelial cells with a higher responsiveness to growth stimuli or directly regulate cell multiplication. This interpretation is consistent with the most recent view of CAM's function, which encompasses not only cell–cell aggregation within tissues, but also regulation of cell multiplication (Edelman and Crossin, 1991; Sporn et al., 1995), cell differentiation (Kemler et al., 1977; Edelman and Crossin, 1991; Takeichi, 1995), migration and three-dimensional organization within tissues during morphogenesis (Crossin et al., 1985; Friedlander et al., 1989; Edelman and Crossin, 1991; Takeichi, 1991, 1995; Larue et al., 1996), as well as tissue remodeling during wound healing (Watt and Jones, 1993; Krushel et al., 1995), and tumoral transformation in adult organisms (Parish et al., 1987; Hynes, 1989). Thus, CAMs should be regarded as transducers of diverse morphoregulatory messages within cell collectives.

Interestingly, despite its functional involvement in cell adhesion, Ep-CAM does not share significant homology with any of the well-characterized families of adhesion molecules such as CAMs of the immunoglobulin superfamily, cadherins, integrins, and selectins. However, the presence of two EGF-like domains comprised between a

cysteine-rich and cysteine-poor domain in the extracellular sequence of Ep-CAM confers some structural homology with members of the *Drosophila Notch* and the *Caenorhabditis elegans Lin* family of proteins. Notably, both *Notch* and *Lin* gene products are involved in cell–cell signaling pathways controlling cell fate decisions during development (Artavanis-Tsakonas et al., 1995; Katz et al., 1995). With respect to the common structural folding of EGF-like domains shared by many proteins involved in pleiotropic and developmental effects, Ep-CAM also shares some homology with tissue plasminogen activator, regulating proteolysis of extracellular matrix during cell growth and migration (Blasi et al., 1987; Appella et al., 1987). Thus we propose that Ep-CAM belongs to a novel family of epithelial-specific cell surface receptors mediating adhesion and delivering growth/developmental signals. Our findings provide strong evidence for a morphoregulatory role of Ep-CAM in pancreatic islet development.

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Molecular Cloning and Characterization of a Human Adenocarcinoma/Epithelial Cell Surface Antigen Complementary DNA

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ABSTRACT

A human adenocarcinoma-associated antigen (KSA) defined by the monoclonal antibody KS1/4 has become the focus of several site-directed strategies for tumor therapy. KSA, a 40,000 Da cell surface glycoprotein antigen, is found at a high density in all adenocarcinomas examined to date and in corresponding normal epithelial tissues. Here we describe the cloning and sequencing of overlapping complementary DNA clones which encode the entire KSA as expressed in UCLA-P3, a human lung adenocarcinoma cell line. We have deduced the 314-amino acid sequence and have compared it to the N-terminal amino acid sequence data of the affinity-purified antigen. The KSA is synthesized as a 314-residue-long preproprotein that is then processed to a 232-residue-long antigen. KSA appears to have a single transmembrane domain of 23 residues that separates the highly charged 26-residue cytoplasmic domain from the extracellular domain. The N-terminal region of the propeptide is rich in cysteines and contains three potential *N*-glycosylation sites. Computer-assisted analyses at both the DNA and protein levels have found no significant similarities of this protein to known sequences, but a GC-rich 5′ terminus is evident. Northern blot analysis shows that transcription of KSA can be detected in RNA isolated from normal colon but not in RNA isolated from normal lung, prostate, or liver.

INTRODUCTION

The development of hybridoma technology has provided numerous monoclonal antibody probes which appear to define tumor-associated gene products. MoAbs[2] to human tumor-associated antigens are currently being widely studied as possible diagnostic and therapeutic reagents (1–8). To date, however, relatively little information is available on the molecular nature of the targeted antigens. However, in some cases, structural information of human tumor-associated antigens has been deduced from cDNA analysis (9–11). Such structural information could help the basic understanding of tumor cell biology and may aid in the design of novel antigen directed monoclonal antibodies.

Adenocarcinomas of the lung, colon, prostate, and breast encompass some of the major forms of solid tumors. With this in mind, the adenocarcinoma-associated antigen recognized by KS1/4 (12) has been under investigation as a target for site-directed therapy approaches with covalent monoclonal antibody oncolytic drug conjugates (13–16). To date this antigen has been found on all adenocarcinoma cells tested and in various corresponding normal epithelial cells. In this study, we report on the molecular cloning of this unique epithelial malignancy/epithelial tissue glycoprotein from the human lung adenocarcinoma cell line, UCLA-P3, and discuss initial studies on its expression.

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[2] The abbreviations used are: MoAb, monoclonal antibody; cDNA, complementary DNA; KSA, 40,000-Da cell surface glycoprotein antigen; SDS, sodium dodecyl sulfate; SSC, standard saline-citrate.

MATERIALS AND METHODS

Construction and Screening of a λgt11 cDNA Library. Total RNA was prepared from UCLA-P3 cells grown in culture. The RNA was extracted with guanidinium thiocyanate (17) and polyadenylated RNA was isolated using oligo(dT) deoxythymidylate cellulose column chromatography. Double stranded cDNA was synthesized using the RNase H procedure (18) and size selected on an agarose gel. DNA over 700 base pairs long was methylated and ligated to synthetic *Eco*RI linkers and then inserted into *Eco*RI-digested λgt11 DNA.

Antibody and Radiolabeled Probe Screening. The λgt11 library was screened at approximately 60,000 plaques/100-mm plate by the method of Young and Davis (19). KS1/4 monoclonal antibody or a primary rabbit polyclonal antibody to KSA was used for initial screening. The secondary biotinylated horse antibody to mouse IgG or a goat antibody to rabbit IgG and avidin-conjugated horseradish peroxidase was purchased from Vector Laboratories. The positive plaque was cycled through four rounds of screening. Plaque hybridization with [^{32}P]RNA probes generated from an SP6 vector were carried out essentially as described by Melton *et al.* (20).

Blot Hybridization. The RNAs were electrophoresed through a 1.5% agarose/6% formaldehyde gel, stained with acridine orange, and transferred to a Genescreen nylon membrane. RNAs were prehybridized according to the Genescreen manual in 10 ml of 50% formamide, 10 × Denhardt's, 0.05 M Tris (pH 7.5), 1 M NaCl, 0.1% sodium pyrophosphate, 0.1% SDS, 10% dextran sulfate, and 100 μg/ml *Escherichia coli* DNA for 6 h at 42°C with agitation. Nick-translated *Sst*II-*Bgl*II cDNA fragment of Ag1338 (3×10^6 cpm/ml; specific activity, 3.9×10^7 cpm/μg) was then added and the RNAs were hybridized overnight at 42°C with agitation. Washes were carried out with constant agitation as follows: 2 times for 5 min each in 2 × SSC at room temperature; 2 times for 30 min each in 2 × SSC and 0.5% SDS at 65°C; and 1 time for 5 min in 0.1% SSC at room temperature.

Recombinant Antigen Synthesis and Detection. Cell pellets of A_{590} = 1 were prepared from cultures grown at 32°C for uninduced pL and at 42°C for induced pL. Lysates were prepared by incubation on ice for 1 h in lysis mix [1 mM EDTA (pH 8.0), 6 μg/ml DNase, and 170 μg/ml lysozyme] and subsequently carried through three cycles of freeze/thaw. Cell debris was removed by centrifugation, lysates were dot blotted onto nitrocellulose, and the blot was probed with a rabbit polyclonal antibody raised against purified KSA (21).

RESULTS

Isolation and Characterization of Antigen cDNA Clones. KSA cDNAs were isolated from a UCLA-P3 λgt11 library of approximately 291,000 individually derived clones. No positive plaques were obtained when this library was screened with the KS1/4 MoAb, but rescreening with a rabbit polyclonal antibody raised against affinity-purified antigen (21) did yield a single positive phage which was plaque purified and found to contain a 249-base pair insert (Ag1). Ag1 was radiolabeled and used to rescreen the library for a larger insert clone. Five positive plaques were found, the largest of which was 1338 base pairs long (Ag1338). The 750-base pair 5′ region of Ag1338 was then radiolabeled and used to probe a RNA blot (22, 23) of UCLA-P3 and normal human liver mRNAs. The 750 base pair probe hybridized to a single UCLA-P3 mRNA species of approximately 1700 bases but did not hybridize to normal human

liver mRNA (data not shown). It, therefore, appeared unlikely that Ag1338 represented a full length mRNA sequence. This same probe was then used to screen the library a third time and detected a positive plaque containing a 933-base pair insert (Ag933) that overlapped Ag1338.

Fig. 1 shows the complete nucleotide sequence of the initial clone, Ag1, and the two overlapping clones, Ag1338 and Ag 933. Together these clones code for an antigen sequence of 1516 base pairs. If one assumes a typical mRNA to have a 200- to 250-nucleotide polyadenylated tail (24), this composite sequence should closely represent full length KSA mRNA. The sequence contains a 405-nucleotide 3′ noncoding region, excluding the polyadenylated tail, which contains the AATAAA polyadenylation signal (25) (1486 to 1491) 13 nucleotides upstream from the polyadenylated tract. The 5′-untranslated region is GC rich similar to the transcription initiation regions of many genes (26). This region contains an untranslated sequence that is 78.9% GC and a proposed signal peptide sequence that is 71.4% GC. The remainder of the coding region is 41.5% GC (Fig. 2).

There is a single long open reading frame that codes for a [illegible] peptide of 314 residues. Only one AUG translation [illegible] codon is present at the 5′ end of the cDNA. This methionine codon is in frame with the remainder of the peptide and lies in a nucleotide sequence that closely corresponds to a consensus translation initiation site (27). It is followed by a hydrophobic signal-like sequence. Inspection of the deduced amino acid sequence suggests a number of potential sites for cleavage of the signal peptide (28); the most likely site is after residue 23 (alanine).

The 23-residue hydrophobic carboxy-terminal domain (residues 266 through 288) represents a potential transmembrane region that separates a highly charged 26-residue cytoplasmic domain from a potential 242-residue extracellular domain (Fig. 1).

The extracellular domain contains a region moderately rich in cysteine residues (12 of 109). The number of disulfide bonds is not known although some must be present since the reduced antigen has a molecular mass of 36 kDa by SDS-polyacrylamide gel electrophoresis, in contrast to the 40–42-kDa mass of the unreduced form. This apparent disulfide-dependent conformation must also provide the epitope recognized by MoAb KS1/4, since the reduced molecule is unreactive with this antibody. The extracellular domain also contains three possible sites for asparagine-linked glycosylation.

KSA appears to be synthesized as a preproantigen peptide. *N*-terminal sequence analysis of affinity purified KSA, prepared from UCLA-P3 cells grown either as a nude mouse xenograft tumor or in tissue culture, starts with residue 82 (alanine) of the cDNA-deduced peptide sequence (21).

Expression of the KSA. In an attempt to show that the cDNA codes for KSA as recognized by the KS1/4 MoAb, an *E. coli* expression vector was constructed that contains the predicted mature KSA sequence (residues 82 through 265). This protein was synthesized under the control of the λpL promoter and immunologically detected as dot blots using the previously mentioned rabbit polyclonal antibody. As seen in Fig. 3, the polyclonal antibody recognizes only the KSA construction in which the pL promoter was induced. It does not recognize those constructions in which the pL promoter was uninduced or those containing an unrelated gene in an identical vector that was induced. The KS1/4 MoAb failed to recognize the *E. coli*-synthesized antigen (data not shown) which probably represents an inability of *E. coli* to properly fold and/or posttranslationally modify KSA.

Blot Hybridization of RNA from Different Tissues. The KSA cDNA was also used to survey various tissue for the presence of this antigen. A 1112-nucleotide *Sst*II to *Bgl*II fragment which encompasses most of the coding region of KSA was nick-translated and used to probe an RNA blot of normal human colon, lung, and prostate; CaCO2 (a human colon carcinoma); and UCLA-P3 RNAs. As shown in Fig. 4, a positive signal was

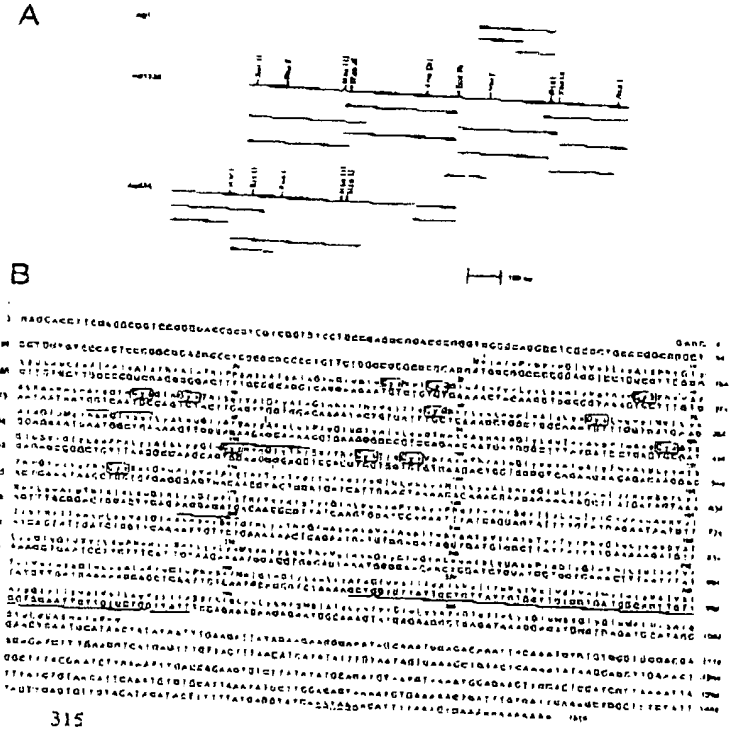

Fig. 1. *A*, sequencing strategy for cDNA clones. *Arrows*, direction and extent of sequencing. Restriction enzyme cleavage sites refer to fragments that were subcloned into M13 vectors for dideoxy sequencing and subsequent manipulation. *B*, composite nucleotide sequence obtained from Ag1338 and Ag933 overlapping clones and deduced amino acid sequence. Cysteinyl residues are *boxed* and potential *N*-glycosylation sites are *overlined*. *Arrow*, cleavage site that gives rise to mature KSA; *heavy line*, potential transmembrane region; *dashed line*, polyadenylation signal sequence.

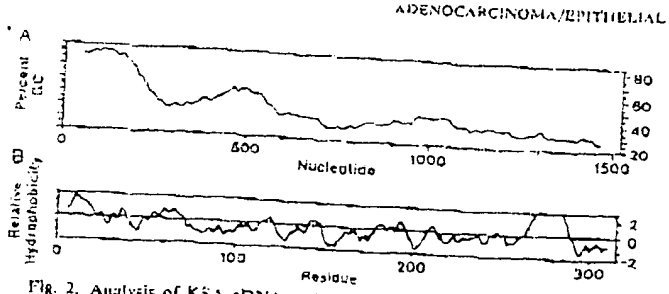

Fig. 2. Analysis of KSA cDNA and protein structure. *A*, percentage of GC content averaged over 100 nucleotides along the 1516-base pair DNA. *B*, Kyte-Doolittle hydrophobicity averaged over nine amino acids along the predicted 314-amino acid protein sequence (38).

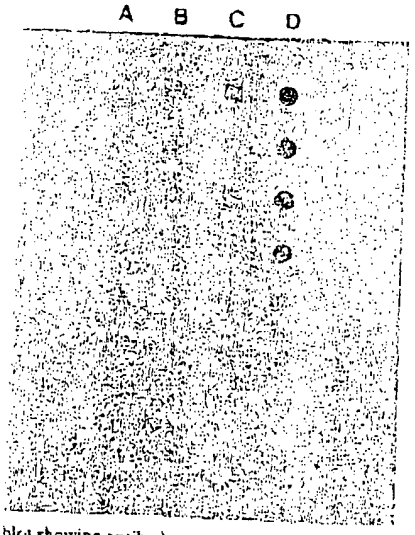

Fig. 3. Dot blot showing antibody recognition of *E. coli*-synthesized KSA (14). Each *column* represents serial 10-fold dilutions from *top* to *bottom*. *A*, lysate of uninduced pL expression plasmid coding for a protein unrelated to KSA; *B*, induced pL expression of *A*; *C*, uninduced pL expression of KSA; *D*, induced pL expression of KSA.

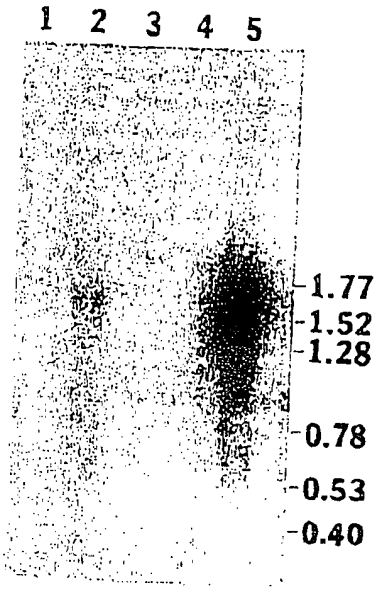

Fig. 4. Northern blot hybridization (15) of KSA cDNA to 10 μg of total RNA isolated from CaCO2 cells (*Lane 1*), normal human colon (*Lane 2*), prostate (*Lane 3*), lung (*Lane 4*), and UCLA-P3 cells (*Lane 5*). Autoradiograph shown is from a 3.5-h exposure. RNA marker sizes are shown at right in kilobases.

detected with normal human colon and CaCO2 but not with lung or prostate RNAs. These results are consistent with immunoperoxidase studies (29) that have shown the KS1/4 antigen to be highly expressed in normal colon epithelial cells but expressed only at low levels in lung or prostate which contain only a fraction of epithelial cells.

DISCUSSION

We present here the molecular cloning of KSA from the human adenocarcinoma cell line UCLA-P3. This work represents one of only several reports characterizing monoclonal antibody-defined cell surface antigens and the first epithelial malignancy integral membrane glycoprotein. KSA shows structural features that are common to a number of cell membrane proteins such as a cysteine-rich region, *N*-glycosylation sites, a hydrophobic transmembrane domain, and a highly charged cytoplasmic anchorage domain (30–32). Computer-assisted analyses at both the nucleotide and amino acid levels found no significant homology of KSA with known sequences. Thus, the KSA sequence provides one of the first detailed descriptions of the primary structure of a membrane-bound tumor-associated antigen.

What emerges from this study is that KSA is a cell surface protein that is synthesized as a 314-residue preproprotein that is processed to a 232-residue antigen. The alanine 82-processing site is proceeded by two arginine residues which could serve as a trypsin-like proteolytic cleavage signal. At this time there is no biochemical information on KSA processing in normal cells or other adenocarcinoma-derived cell lines. Processing removes 7 of the 12 cysteine residues as well as one of the three potential *N*-glycosylation sites found in the proantigen sequence. The mature 26,340-Da antigen peptide appears to be highly glycosylated since based on polyacrylamide gel electrophoresis the molecular mass is approximately 40,000 Da (12). Presumably both the 183-residue extracellular domain and the sugar moiety contain potential antibody-binding epitopes.

The proposed cytoplasmic domain has nine positively charged and five negatively charged residues giving it a net positive charge of 4 which is similar to the net positive charge of 6 found in the abbreviated cytoplasmic domain of the 55,000-Da interleukin 2 receptor protein (33). There is a single serine residue in the cytoplasmic domain that may serve as a phosphorylation site. None of the above-described characteristics, however, has given us any insight into the biological role of this protein. In our initial tissue survey it appears that only colon, a tissue rich in epithelial cells, has detectable levels of KSA RNA transcription. *In situ* hybridization techniques will be attempted to give a more accurate picture of transcriptionally active tissue.

With the cloning of KSA, structural and functional studies of this glycoprotein are now possible. The cloning of KSA will also facilitate the generation of MoAbs directed at epitopes other than that recognized by KS1/4 and facilitate the possibility of expressing sufficient quantities of KSA in mammalian cells for studies of the molecular interactions between KSA and the KS1/4 MoAb. In addition, the relationship of the KSA glycoprotein to other tumor associated antigens with similar molecular characteristics can now be explored (34–37). In this regard, preliminary evidence suggests that antibodies 17-1A (34) and AUA1 (35) do react with the affinity-purified KSA from tumor xenografts which was proven to be homogenous by

N-terminal amino acid sequence.[3] These data may unify both preclinical and clinical data of various antibodies based on the utility of KSA as a target for immunotherapy.

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Immunology

IgG2a monoclonal antibodies inhibit human tumor growth through interaction with effector cells

(macrophages/cytotoxicity/*nude* mice)

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ABSTRACT Monoclonal antibodies of IgC2a isotype specifically inhibited growth of human tumors in *nude* mice. Twenty-three monoclonal antibodies of other isotypes showed no tumoricidal reactivity. Complement depletion of *nude* mice had no effect on tumor suppression by monoclonal antibody. The role of T and killer cells as mediators of the monoclonal antibody effect in *nude* mice was virtually excluded. On the other hand, macrophages were strongly incriminated as effector cells because silica treatment of *nude* mice abolished the tumoricidal effect of monoclonal antibody. IgG2a monoclonal antibody-dependent macrophage-mediated cytotoxicity assays with human tumor cells in culture resulted in specific lysis of tumor cells.

We have previously reported that hybridoma-derived mouse monoclonal antibodies (MAbs) effectively inhibit growth of human melanomas and colorectal carcinomas in *nude* mice (1, 2). Growth of mouse leukemic cells was also inhibited by MAbs (3–6). The effective MAbs were shown to be of IgG isotype, whereas IgM antibodies of the same binding specificity were less effective (5) or totally ineffective (4, 6, 7). In addition, it was shown (8) that IgG2a but not IgG2b mouse polyclonal antibodies suppressed the growth of murine tumors.

In the present study we tested a large number of monoclonal antibodies produced against human tumors of various origins for their tumor growth-inhibiting properties in *nude* mice. Within the context of this study we have also investigated tumor growth suppression related to a specific isotype of MAb. In parallel studies, MAbs with tumoricidal effects in *nude* mice were tested for cytotoxic effects against tumor targets in tissue culture.

MATERIALS AND METHODS

Human Cell Lines. We included three colorectal carcinomas (CRC), one pancreas carcinoma (PC), two melanomas (MEL), two lung carcinomas (LC), one breast carcinoma (BC), and one epidermoid carcinoma (EC). Most of these cell lines have been described elsewhere (9, 10). The PC line Capan II was kindly provided by T. Fogh (Memorial Sloan–Kettering Cancer Center, New York), and the EC line Hep 2 was obtained from the American Type Culture Collection (Rockville, MD).

Mouse MAbs. The MAbs used in this study are described in Table 1. MAb $H_2$4B5 was kindly provided by W. Gerhard (The Wistar Institute).

Effect of MAb Administration on Tumor Growth in *nude* Mice. Thymus-deficient *nude* mice (*nu/nu*, BALB/c background) 6–8 weeks old (unless otherwise stated) were implanted with tumor cells and injected with anti-tumor MAb or control anti-influenza virus MAb, both in ascitic fluid, as described (2).

Table 1. MAbs used in the present study

Mice immunized with	Binding specificity of MAb*	MAb	Ref.
CRC	GIC	17-1A	11
		19-9	9
		38a	9
		$C_4$14-72	12
		$C_4$20-32	†
MEL	MEL	Nu4-B	1
	MEL‡	37-7	10, 13
LC	LC, CRC, BC	16B-13	14
Influenza virus	Influenza virus	$H_2$4B5	15

GIC, gastrointestinal carcinoma.
* Determined in radioimmunossay (RIA).
† Unpublished results.
‡ Also shows DR antigen specificity.

In some experiments, F(ab')$_2$ fragments of anti-CRC MAb 17-1A, prepared by pepsin digestion of the intact antibody molecule, were injected into tumor-bearing mice. Tumor volumes were monitored as described (2).

Immunosuppressed Mice. C57BL mice were thymectomized, irradiated, and bone marrow-reconstituted as described (16). The effect of MAb treatment on tumor growth in these mice was investigated as described for *nude* mice (see above).

Treatment of *nude* Mice with Anti-Interferon Serum. The anti-interferon serum was kindly provided by I. Gresser (Institut de Recherches Scientifiques Sur le Cancer, Villejuif, France) and was injected 5 days and 1 hr before the injection of tumor cells and MAb as described (17).

Treatment of *nude* Mice with Silica. To study the effect of silica treatment on suppression of tumor growth by MAb, *nude* mice were injected intravenously with 3 mg of silica particles (Whittaker, Clark and Daniels, South Plainfield, NJ; average particle size, 1.5 μm) in 0.3 ml of phosphate-buffered saline (P_i/NaCl) 6 hr before the injection of tumor cells and MAb. The silica preparation was exposed to ultrasonic vibration and subsequent Vortex mixing before injection (18).

Peritoneal Exudate Cells. Ten- to 12-week-old CBA mice were inoculated intraperitoneally with 1 ml of thioglycollate

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Abbreviations: ADLC, antibody-dependent lymphocyte-mediated cytotoxicity; ADMC, antibody-dependent macrophage-mediated cytotoxicity; BC, breast carcinoma; CRC, colorectal carcinoma; EC, epidermoid carcinoma; GIC, gastrointestinal carcinoma; K cells, killer cells; LC, lung carcinoma; MAb, monoclonal antibody; MEL, melanoma; P_i/NaCl, phosphate-buffered saline; PC, pancreas carcinoma; RIA, radioimmunoassay.

broth (Difco). Four days later, peritoneal exudate cells were collected in 10 ml of P_i/NaCl containing 100 units of heparin.

Monoclonal Antibody-Dependent Macrophage-Mediated Cytotoxicity (ADMC) Assays. *Colony formation inhibition assay.* Peritoneal exudate cells were pelleted and resuspended to 5×10^5 cells per ml in Eagle's minimal essential medium containing 10% fetal calf serum. This medium was used throughout the assay. One milliliter of the cell suspension was put in each well of a 24-well tissue culture plate (Linbro FB-16-24-TC). The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere for 2 hr to allow them to attach to the plate. Cells were then washed three times with 1 ml of P_i/NaCl. The adherent cell population consisted of 85% phagocytic cells (average of 10 experiments) as determined by zymosan particle incorporation and nonspecific esterase staining (19, 20). These adherent cells will be referred to as macrophages. One milliliter of MAb in ascitic fluid diluted 1:100 with medium was added to triplicate wells containing macrophages, followed by the addition of human tumor target cells in 1 ml of medium (effector-to-target cell ratio, 50:1). Control wells received macrophages, target cells, and medium or anti-influenza virus MAb. After a 7-day incubation, cells in each well were trypsinized, suspended in 1 ml of a 0.3% agar (agarose, Marine Colloids Division of FMC, Rockland, ME) solution in medium and transferred to a 30-mm Petri dish containing a 0.5% agar bottom layer. The colonies per dish were counted under a light microscope after a 2-week incubation. The colonies growing on agar were solely derived from human tumor cells because macrophages did not form colonies. Colony formation inhibition by MAbs was calculated as percent of controls treated with medium alone.

[methyl-^3H]*Thymidine release assay.* This assay was performed essentially as described by Mantovani *et al.* (21). Briefly, target cells labeled with [*methyl*-^{3}H]thymidine (5 μCi per 10^6 cells; specific activity 82.6 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) were added to wells of microtiter plates containing macrophages (effector-to-target cell ratio, 50:1), followed by the addition of MAbs or medium (controls). After a 3-day incubation of the plates at 37°C in a humidified CO_2 incubator, half of the supernatant was removed for determination of radioactivity. Spontaneous and maximal release of radioactivity by the targets were determined as described (21) and the percentage of specific lysis was calculated (22).

Antibody-Dependent Lymphocyte-Mediated Cytotoxicity (ADLC). The activity of spleen lymphocytes derived from adult and neonatal *nude* mice and from immunosuppressed C57BL mice in ADLC against ^{51}Cr-labeled target cells was measured as described (22).

RESULTS

Effect of MAb on Established Tumors in *nude* Mice. The tumor-suppressive effect of MAb 17-1A administered simultaneously with CRC cells has been described before (2). The effect of delayed administration of MAb on CRC tumor growth was studied in *nude* mice implanted with CRC cells and injected 4 or 7 days later with MAb 17-1A. Tumor growth was effectively inhibited ($P < 0.05$, t test, when compared to controls), and histological examination showed the absence of viable tumor cells within 2 days after MAb treatment.

Isotypes of Tumor-Inhibiting MAbs. Thirty-three MAbs that bound in RIA to human tumor cells of various origins were tested *in vivo* for tumor growth inhibition activity. Of these, five inhibited growth of tumors in *nude* mice. All five antibodies are of IgG2a isotype, whereas the noninhibiting MAbs belonged to five other immunoglobulin classes (IgG1, IgG2b, IgG3, IgM, and IgA).

Specific destruction of tumor cells by the five IgG2a MAbs is represented in Table 2. The MAb 17-1A, which binds in RIA only to tumor cells of GIC (11), destroyed CRC tumors only and did not destroy MEL or LC. Two other anti-GIC MAbs also destroyed CRC. The MAb 16B-13, which binds in RIA to cells of LC, CRC, and BC (14), suppressed the growth of these tumors but not others. MAb 37-7, which binds to MEL and also shows DR antigen specificity (10, 13), suppressed the growth of DR-antigen-positive MEL but not of Hep 2 cells, which also express this antigen.

Destruction of Tumor Cells in *nude* Mice. Although none of the IgG2a MAbs shows complement-dependent cytotoxicity of tumor cells in culture (results not shown), they bind *nude* mouse complement. To investigate the possible role of complement in tumor suppression by MAbs, adult *nude* mice were implanted with CRC cells, injected with 17-1A MAb, and treated daily with cobra venom factor (Cordis Laboratories, Miami, FL) to deplete the mice of complement as described by Oldstone and Dixon (23). [These experiments were performed by M. Oldstone (Scripps Clinic and Research Foundation, La Jolla, CA) and Z. Steplewski (The Wistar Institute).] Although complement component C3 was undetectable after administration of cobra venom factor, tumor destruction by MAb was as effective as in untreated mice (results not shown). These results suggested involvement of effector cells in the tumor destruction by MAb and that these effector cells must express Fc receptors, because $F(ab')_2$ fragments of the antibody do not show tumor-suppressive activity. It is rather unlikely that T cells would function as effector cells in athymic *nude* mice, and therefore we have investigated the role of killer (K) cells as effector cells. Their role as effector cells was also unlikely, for although K cell activity in adult *nude* mice is high, the MAb inhibited tumor growth in 10- to 12-day-old *nude* mice, spleens of which showed no K cell activity (Fig. 1). Furthermore, treatment of adult *nude* mice with an anti-interferon serum that suppresses K cell activity did not affect inhibition of tumor growth by MAb (results not shown). We were also able to grow CRC cells in immuno-

Table 2. Inhibition of human tumor growth in *nude* mice by MAbs of IgG2a isotype

Mice immunized with	Binding specificity of MAb	MAb	Human tumors implanted into *nude* mice	Mice with suppressed tumor growth
CRC	GIC	17-1A	CRC-1	54/54
			CRC-2	4/4
			MEL-1	0/5
			LC	0/8
		$C_4$14-72	CRC-1	12/12
		$C_4$20-32	CRC-1	11/11
LC	LC, CRC, BC	16B-13	LC-1	5/5
			LC-2	5/5
			CRC	4/4
			BC	5/5
			MEL-1	0/5
MEL	MEL	37-7	MEL-1	6/6
			MEL-2	4/4
			EC	0/5
			CRC	0/5

Immediately after subcutaneous implantation of 5×10^6 tumor cells, mice were treated intraperitoneally with approximately 400 μg of MAb (in ascitic fluid) daily for 5 days and then twice more on the 8th and 12th days. Results are presented as the ratio of mice with suppressed tumor growth to total mice treated.

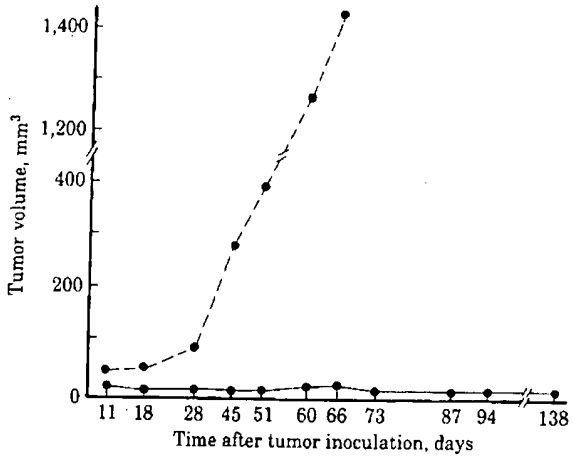

FIG. 1. Inhibition of CRC tumor growth in 10- to 12-day-old *nude* mice by MAb 17-1A. Ten- to 12-day-old *nude* mice were inoculated with CRC and treated with MAb 17-1A (●——●) or control anti-influenza virus antibody H_24B5 (●- - -●) as described in the legend to Table 2. Six mice were included per treatment group. MAb 17-1A significantly ($P < 0.05$, t test) inhibited tumor growth. Spleen cells of neonatal *nude* mice showed no K cell activity (percent specific lysis with MAb 17-1A = 0.3) in ^{51}Cr release assays (22), in contrast to the high activity in spleens of adult *nude* mice (percent specific lysis with MAb 17-1A = 25).

suppressed C57BL mice. Spleen cells of these mice showed no K cell activity. The MAb effectively inhibited tumor growth in these mice.

Effect of Silica on Inhibition of Tumor Growth in *nude* Mice. As shown in Fig. 2, treatment of *nude* mice with silica abolished the suppressive effect of MAb on the growth of CRC, because tumors in mice treated with silica and MAb 17-1A grew progressively at a rate comparable to that observed in mice treated with anti-influenza virus MAb. Because silica particles of 1.5 μm size seem to primarily inactivate macrophages (18), we have investigated the role of those cells as effector cells in ADMC.

Monoclonal Antibodies in ADMC. Peritoneal macrophages were obtained from CBA mice and mixed with CRC cells and with each of the seven MAbs listed in Table 3, and the mixtures were incubated for 7 days. Destruction of CRC cells was determined by colony inhibition assays and by lysis. Two of the antibodies, 17-1A and 16B-13, which bind in RIA to CRC cells (11, 14), destroyed CRC cells in ADMC. These two MAbs also suppressed the growth of the same CRC tumors in *nude* mice. The three other IgG2a MAbs, which bind either to MEL (37-7 and Nu4-B) or to influenza virus (H_24B5), had no effect in ADMC. The IgG1 MAb 19-9, which binds CRC cells and destroys them in ADMC, had no suppressive effect on the growth of these tumors in *nude* mice. Finally, the MAb 38a of IgM isotype binds CRC cells, but did not show ADMC against those cells and did not suppress the growth of CRC in *nude* mice.

As shown in Table 4, MAb 17-1A, which binds in RIA to tumors of GIC only, mediated an ADMC effect against cells of GIC (three CRC and one PC). MEL or LC cells, which do not bind MAb 17-1A, were not lysed in ADMC.

Similar results were obtained with macrophages obtained from BALB/c and *nude* mice (results not shown).

Role of Granulocytes in ADMC. We were able to demonstrate that granulocytes were not involved in the ADMC reactions shown in Tables 3 and 4: removal of these cells from peritoneal exudate cells by Ficoll purification prior to the assay had no effect on ADMC.

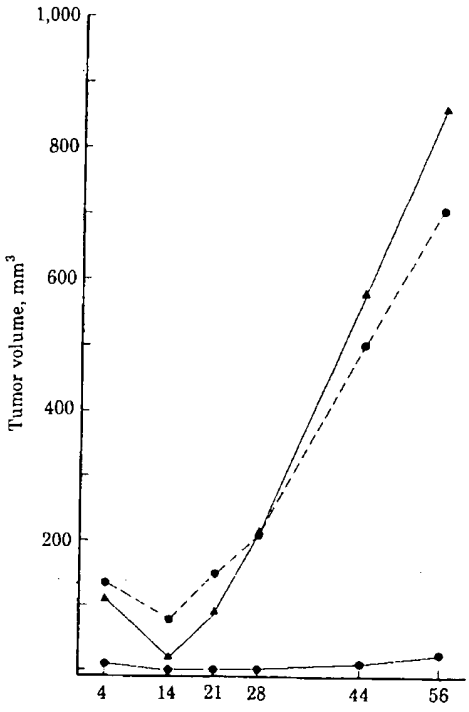

FIG. 2. Effect of silica on MAb-mediated tumor growth inhibition in *nude* mice. Six *nude* mice were injected with CRC cells and MAb 17-1A (●——●) or anti-influenza control antibody (six mice) H_24B5 (●- - -●), as indicated in the legend to Table 2. Six mice that had been injected with CRC cells and MAb 17-1A were treated with silica 6 hr later (▲——▲). Silica treatment abolished tumor suppression by MAb 17-1A; tumor volumes in these animals did not differ significantly ($P > 0.1$) from control tumors.

Interaction of MAb with Target and Macrophages. CRC cells (1×10^4) were exposed to 4 mg of MAb 17-1A, incubated for 1 hr at room temperature, washed once, and then exposed

Table 3. Effect of MAbs of various binding specificities on growth of CRC in culture and in *nude* mice

Isotype	Binding specificity*	MAb	Colony formation inhibition,† %	Specific lysis,† %	Tumor growth inhibition in *nude* mice
IgG2a	CRC	17-1A	63‡	96‡	+
	CRC§	16B-13	34‡	93‡	+
	MEL¶	37-7	2	−5	−
	MEL	Nu4-B	4	4	−
	Influenza virus	H_24B5	2	8	−
IgG1	CRC	19-9	25‡	91‡	−
IgM	CRC	38a	−2	−7	−

* Determined in RIA.
† Mouse peritoneal macrophages were used as effector cells (effector-to-target cell ratio, 50:1). Results are means of five experiments in line one; all other values in the columns are means of two experiments.
‡ $P < 0.05$ (t test) when compared to controls.
§ Also binds to LC and BC.
¶ Also shows DR specificity.

Table 4. Target cell specificity of MAb 17-1A in ADMC

Target cell	Specific lysis,* %
CRC-1	63 ± 4†
CRC-2	22 ± 1†
CRC-3	52 ± 3†
PC	62 ± 2†
MEL	7 ± 1
LC	2 ± 1

* Mean ± SD for triplicate samples.
† $P < 0.05$ (t test) when compared to controls.

to 5×10^5 macrophages. In a parallel experiment, 5×10^5 mouse macrophages were incubated with 2 mg of MAb 17-1A for 1 hr at 37°C and washed once, and 1×10^4 tumor cells were then added. In both experiments a large excess of MAb was used (approximately 1,000 times the saturation concentration). The results of the experiments (Table 5) indicate that the "armed" macrophages are effective in tumor destruction, whereas exposure of tumor cells to MAb before incubation with macrophages does not necessarily, under the conditions of the experiment, result in tumor cell destruction. These results parallel somewhat those obtained in *nude* mice when 5×10^6 cells of CRC were mixed with 15 mg of MAb 17-1A, incubated for 1 hr at room temperature, washed once, and then implanted into mice. Tumor growth was not suppressed under these conditions (Fig. 3).

Scanning Electron Microscopy. Scanning electron micrographs show macrophages in the presence of specific MAb adhering to tumor target cells that were being destroyed (G. Maul, personal communication); in the absence of MAb, macrophages are not adherent to the intact tumor cells (not shown).

DISCUSSION

The results of the experiments presented in this paper and of those previously described (1, 2) clearly indicate that MAbs are specific and powerful inhibitors of growth of human tumors in *nude* mice. Experimental results also show conclusively that only the MAbs of IgG2a isotype are effective in tumor destruction, whereas MAbs of other isotypes are inactive, even though they show binding activity in RIA to the surface of tumor cells that is similar to the binding observed with IgG2a antibodies. Langlois *et al.* (8) have observed that IgG2a antibodies destroy murine adenocarcinomas in mice, in contrast to IgG2b antibodies of the same specificity, which were inactive.

We have virtually excluded the involvement of complement and of T and K cells as effector cells. The possibility that secondary immune responses of the tumor-bearing mouse raised in the course of MAb treatment are involved in the destruction of the tumors seems unlikely because mice cured by MAb supported the growth of a second homologous tumor implant (results not shown).

Table 5. Effect of prior coating with MAb 17-1A of either CRC cells or mouse macrophages on growth of tumor cells in culture

Cells coated with MAb 17-1A	Colony formation inhibition,* %
CRC†	0 ± 2
Macrophages‡	97 ± 3§

* Results are mean ± SD for triplicate samples.
† CRC cells (1×10^4) were exposed to 4 mg of MAb for 1 hr at room temperature before incubation with 5×10^5 macrophages.
‡ Macrophages (5×10^5) were exposed to 2 mg of MAb for 1 hr at 37°C before incubation with 1×10^4 CRC cells.
§ $P < 0.01$ (t test) when compared to controls.

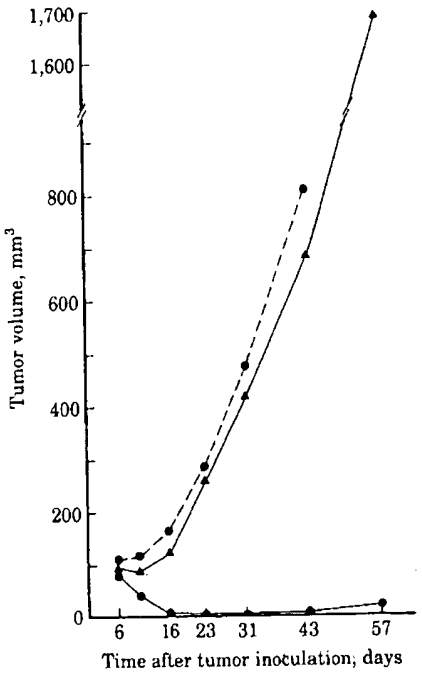

FIG. 3. Effect on tumor growth of coating CRC cells with MAb 17-1A prior to inoculation into *nude* mice. CRC cells (5×10^6) were incubated with 15 mg of MAb 17-1A (▲——▲) or anti-influenza virus MAb $H_2$4B5 (●– – –●), washed once, and injected subcutaneously into *nude* mice. Other animals were injected with 5×10^6 CRC cells subcutaneously and MAb 17-1A intraperitoneally (●——●) as indicated in the legend to Table 2 (six mice were included in each treatment group). The latter treatment resulted in significant ($P < 0.05$, t test) inhibition of tumor growth, whereas the growth of CRC cells precoated with MAb 17-1A did not differ significantly ($P > 0.1$) from the growth of control tumors.

Treatment of *nude* mice with silica of 1.5 μm particle size primarily inactivates macrophages (18), and in such mice the MAbs did not suppress tumor growth. Although there is a slight possibility that other phagocytic cells are also inactivated by silica, macrophages have been singled out as playing a major role in anti-tumor activity in ADMC in the presence of MAb specific for a given tumor target cell. Macrophages have been observed to adhere closely to target cells prior to destruction of these cells; it is impossible, however, to determine whether phagocytosis of tumor cells or their destruction by a cytolytic factor (24) secreted by the activated macrophages occurs.

Destruction of tumor cells in ADMC does not always coincide with suppression of tumor growth in *nude* mice. The IgG1 anti-CRC MAb 19-9 (Table 3), for example, is effective in ADMC but ineffective *in vivo*. Similarly, of six MAbs of IgG2a isotype that react in ADMC with melanoma cells, only one suppressed growth of tumors in *nude* mice.

Apparently, attachment of MAb to Fc receptors of the macrophage is essential to effect tumor destruction; however, the precise requirements for this process are still little understood. From our previous studies it seems that many but not all tumor surface molecules may act as effective target structures. For instance, the four anti-CRC IgG2a MAbs described here are clearly of different fine specificities because three MAbs each precipitated a component of different molecular weight (M. Blaszczyk, personal communication) and one failed to immu-

noprecipitate a tumor cell surface component from CRC cell lysates labeled either with ^{125}I by lactoperoxidase or with [^{75}Se]-methionine. On the other hand, of six anti-melanoma MAbs of IgG2a isotype, only the one reactive with DR antigen suppressed melanoma tumor growth *in vivo* (unpublished observations). Also, correct targeting of a macrophage onto a tumor cell may not be the only requirement for subsequent tumor cell lysis. This is suggested by the observation that the IgG2a MAb with anti-DR specificity suppressed growth of melanomas but not growth of the DR-antigen-expressing Hep 2 cells.

Human buffy coat cells are capable of destroying human tumor cells coated with MAb *in vitro* and prior mixing of tumor cells with antibody-coated human peripheral blood lymphocytes will prevent tumor growth in *nude* mice (25). The identity of the human effector cells is, however, a matter of conjecture. Human immunoglobulins of certain isotypes have been shown to bind to rat macrophages (26). It is possible that mouse immunoglobulins bind to Fc receptors on human macrophages and that monoclonal antibodies destroy tumor cells with human macrophages as effector cells. This possibility has far-reaching clinical applications.

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(12) **United States Patent**
Schoemaker et al.

(10) **Patent No.: US 6,444,207 B1**
(45) **Date of Patent: Sep. 3, 2002**

(54) **IMMUNOTHERAPY OF TUMOR WITH MONOCLONAL ANTIBODY AGAINST THE 17-1A ANTIGEN**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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(22) Filed: **Sep. 16, 1994**

Related U.S. Application Data

(63) Continuation of application No. 07/726,640, filed on Jul. 2, 1991, now abandoned, which is a continuation of application No. 07/384,073, filed on Jul. 20, 1989, now abandoned, which is a continuation of application No. 06/883,572, filed on Jul. 9, 1986, now abandoned.

(51) **Int. Cl.**[7] **A61K 39/395**

(52) **U.S. Cl.** **424/156.1**; 530/388.85

(58) **Field of Search** 424/155.1, 174.1, 424/277.1, 156.1, 141.1; 530/388.8, 388.1, 387.1, 828, 388.85; 435/240.27

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(57) **ABSTRACT**

Disclosed is a method for treating a gastrointestinal tumor by administering a murine antibody which specifically binds an epitope of 17-1A antigen.

17 Claims, No Drawings

IMMUNOTHERAPY OF TUMOR WITH MONOCLONAL ANTIBODY AGAINST THE 17-1A ANTIGEN

RELATED APPLICATION

This application is a continuation of U.S. Ser. No. 07/726,640, filed Jul. 2, 1991, now abandoned, which is a continuation of U.S. Ser. No. 07/384,073, filed Jul. 20, 1989, now abandoned, which is a continuation of U.S. Ser. No. 06/883,572, filed Jul. 9, 1986, now abandoned.

BACKGROUND

The tumoricidal activity of the murine monoclonal antibody 17-1A has been characterized in the nude mouse and in humans. See, e.g., Herlyn, D. and Koprowski, H. (1982) "IgG2a Monoclonal Antibodies Inhibit Human Tumor Growth Through Interaction with Effector Cells" *Proc. Natl Acad. Sci.* USA 79, 4761–4765. Several cases have been reported where the administration of Mab 17-1A resulted in a partial or complete regression of metastatic colorectal or pancreatic carinomas. See Sears, H. F. et al. (1984) "Effects of monoclonal antibody immunotherapy on patients with gastrointestinal adenocarcinoma." *J. Biol. Resp. Mod.* 3, 138–150; Sears, H. F., et al., "Phase II Clinical Trial of a Murine Monoclonal Antibody Cytotixic for Gastrointestinal Adenocarcinoma" (1985) *Cancer Res.* 45: 5910–5913. Generally, the antibody has been administered as single administration of 500 μg or less.

SUMMARY OF THE INVENTION

This invention pertains to a method of immunotherapy of gastrointestinal tumors employing multiple, high doses of murine monoclonal antibody against the gastrointestinal tumor-associated antigen 17-1A. The method comprises administering to a patient afflicted with gastrointestinal tumor, murine monoclonal antibody against the antigen 17-1A in multiple, sequential doses of about 100 mg or more for a total overall dose of from about 0.1 to about 5 grams. Each dose of the murine antibody can be administered at one to three day intervals up to weekly intervals to achieve and maintain a "continuous" high level of circulating antibody. Mixtures ("cocktails") of two or more murine anti-17-1A antibodies can be given. The multiple, high dose therapy can be performed as adjuvant therapy to chemotherapy, radiotherapy or surgery.

The high dose murine antibody therapy is well tolerated in patients. Further, the anti-murine antibody response which generally develops in treated humans, surprisingly does not alter significantly the plasma half life of the murine antibody on repeat administration. Thus, high blood levels of antibody can be achieved with sequential injections of high doses to enhance transit of the antibody from intravascular space into the tumor bed and thus provide higher concentrations of the therapeutic antibody to the locus of action.

DETAILED DESCRIPTION OF THE INVENTION

This invention pertains to therapy of gastrointestinal tumors with repeated, high dose of murine antibody against the 17-1A antigen associated with most gastrointestinal tumors. This therapeutic approach is based upon several findings. Murine anti-17-1A antibody administered in multiple, high dose is generally well-tolerated by patients. The most common side effect is mild gastrointestinal symptoms. Allergic responses, however, do limit repeat therapy in some patients. In addition, although a human anti-murine antibody response is generally evoked by the murine antibody, the response does not drastically effect the pharmacokinetics of the administered murine antibody. This indicates that sequential, high doses of antibody can be given to achieve and maintain a continuous high plasma level of antibody. Maintaining a high circulating level of antibody optimizes transit of the antibody from intravascular space into the tumor, thereby enhancing access of the antibody to the tumor for more effective anti-tumor action. Further, sustained, high blood levels lead to a prolonged, higher concentration of antibody at the locus of action for more effective antibody dependent cell mediated cytolysis of the tumor cells.

According to the method of this invention, murine antibody against the 17-1A antigen is administered to patients afflicted with gastointestinal tumors in multiple doses of about 100 mg or more, preferably about 400 mg –1 gram, for a total dose of about 0.1 to 5 grams, preferably 1 to 5 grams. The antibody is administered parenterally preferably by intraveneous infusion. The antibody is generally administered suspended in a physiologically acceptable vehicle e.g. normal saline. The antibody doses can be given over intervals of 1–3 days to intervals of about a week. The dose regimen for an individual patient will depend, inter alia, on the patient's clinical status and on his ability to tolerate the dose without detrimental allergic or anaphylactic reaction. The objective is to provide antibody at fractional doses which yields a sustained, high plasma level of antibody over the course of therapy to provide increased access of the antibody to the tumor site.

Murine antibodies against 17-1A can be administered individually or in mixtures (cocktails) of two or more murine anti-17-1A antibodies. Preferably, anti-17-1A antibody having different epitopic specificity for 17-1A is employed in the combination in order to increase anti-tumor activity in an additive or synergistic fashion. Murine antibodies can be selected from the original 17-1A antibody or other murine antibodies which recognize similar or different epitopes of the 17-1A antigen, such as the M72, M74, M77 and M79 antibodies described below.

Murine antibody against 17-1A antigen can be used in passive immunotherapy of tumors of the gastrointestinal tract with which the 17-1A antigen is associated. Examples are gastrointestinal adenocarcinoma, colorectal carcinoma and pancreatic carcinoma. The murine antibody treatment can be adjuvant to other forms of therapy, including chemotherapy, radiotherapy and/or surgery. In particular, murine antibody therapy can be useful as adjuvant therapy directed against micro- or mini-metastases which are not amenable to surgical removal.

The invention is further illustrated by the following exemplification.

Exemplification

A trial was conducted in 20 patients to examine patient tolerance to repeated high doses in 17-1A, to examine its pharmacokinetics on repeated administration and to characterize the human immune response (antibody) to this mouse immunoglobulin.

Patient Population

Twenty patients with gastrointestinal malignancy (17 colon; 2 gastric; and 1 pancreatic) were selected on the basis that they had metastatic disease with small-moderate tumor burden; performance status greater than 70% (Karnofsky scale) and objectively measurable disease. Studies were not done to document 17-1A reactivity with individual patient

tumor specimens. Seven out of 20 patients had received prior chemotherapy while 13/20 had no prior therapy for metastatic disease.

Treatment Protocol

The study was conducted the Clinical Research Unit, Comprehensive Cancer Center, University of Alabama at Burmingham. All antibody infusions utilized a total dose of 400 mg of 17-1A diluted in 250 ml of normal saline infused over 30 minutes with careful monitoring of vital signs. All infusions were preceded by an intravenous test dose of 0.7 mg followed by 30 minutes of monitoring prior to administration of the full dose infusion. The protocol involved the accrual of 4 groups of 5 patients each who would receive progressively increasing numbers of weekly infusions, i.e. Group 1—5 patients—single infusion; Group 2—5 patients with 2 infusions—Day 1 and 8; Group 3—5 patients with 3 infusions—Day 1, 8 & 15; Group 4— 5 patients with 4 infusions—Day 1, 8, 15 & 22. Because of toxicity noted in Group 3 patients, no patients received 4 infusions, but these 5 patients were added to Group 2 and had 2 infusions of therapy (Day 1 & 8). All patients were followed for 6 weeks, following their last infusion with weekly monitoring of urinalysis, liver and renal function, blood counts and clinical evaluation.

Pharmacokinetics

Pharmacokinetic analysis was done on the first 5 patients at the time of their single infusion (no prior exposure to 17-1A), the ten patients who had two infusions were studied at the time of their second infusion (one prior exposure to 17-1A) and the group 3 patients were studied at the time of their third infusion (two prior exposures to 17-1A). For pharmacokinetics, blood samples were drawn prior to infusion, immediately at conclusion of infusion and at ½, 1, 2, 4, 12, 24, 48, 72 hours and 86 hours. Spot samples were drawn at pre-therapy, 1, 24 & 48 hours post-therapy on infusions not undergoing a full pharmacokinetic study to confirm the general pattern of mouse immunoglobulin disappearance. The plasma level of 17-1A was quantitated using a solid phase radiometric sandwich assay utilizing latex beads coated with rabbit anti-mouse gammaglobulin and radiolabeled (^{125}I) affinity purified goat antimouse IgG, $F(ab')_2$. The concentration of 17-1A in plasma was quantitated by the amount of latex particle binding of radiolabeled anti-mouse IgG, $F(ab')_2$ as compared to a standard curve of known concentrations of 17-1A diluted in normal plasma. The sensitivity of this assay was 1.0 ng/ml.

Human Anti-mouse Antibody (HAMA) Response

Serum samples were drawn on each patient prior to each infusion and then weeklyx6. The assay used to determine the presence of human anti-17-1A was a "double antigen" system (Addison, G. and Hale, C., *Horm. Metab. Res.* 3, 59–60 (1971)) using the concurrent incubation of 17-1A coated latex beads, 100 ul of test plasma of 1 ug of radiolabeled (^{125}I) 17-1A (specific activity of 300–400 cpm/ng). The samples were incubated 90 minutes at room temperature and the radioactivity associated with the beads determined by centrifugation of the beads through Percoll as previously described (Lobuglio, A. et al., *New Engl. J. Med.* 309, 459–463 (1983)). The cpm of ^{125}I-17-1A bound to the beads by plasma was converted to ng of 17-1A/ml of plasma by using the known specific activity of ^{125}I-17-1A. This assay obviously detects any molecule with more than one binding site for 17-1A (IgG and IgM). Assay results in normal individuals and cancer patients prior to 17-1A exposure were 5±4 ng/ml plasma (n=54) with values ranging from 0 to 16 ng/ml. Values greater than 20 ng/ml were classified as an antibody response.

17-1A Monoclonal Antibody

The monoclonal antibody was provided by Centocor, Inc. as a purified suspension of 10 mg/ml in normal saline. It was stored at 4° C. prior to use. The protocol was carried out under Centocor sponsored IND (#2168).

Toxicity

The adverse effects of 17-1A administration are summarized in Table 1.

TABLE 1

TOXICITY ASSOCIATED WITH 17-1A INFUSION

I.	Single dose (400 mg) - 5 patients
	3/5 - none
	2/5 - G.I. symptoms
II.	Two weekly doses (400 mg) - 10 patients
	4/10 - none
	5/10 - G.I. symptoms
	1/10 - flushing/tachycardia
III.	Three weekly doses (400 mg) - 5 patients
	3/5 - none
	2/5 - G.I. symptoms and anaphylaxis (third dose)

Ten of 20 patients had no adverse effects including 4 patients who received two infusions and 3 patients who received three infusions. The most frequently observed side effect was gastrointestinal (9/20 patients) with nausea and vomiting (4 patients) or diarrhea with or without cramps (7 patients). The symptoms usually began within an hour of infusion and lasted less than 24 hours. They were of modest-moderate severity and readily controlled with anti-emetics of anti-diarrhea medications. The frequency of gastrointestinal symptoms was not related to the number of 17-1A infusions. One patient had an episode of flushing and tachycardia in the midst of her second infusion which disappeared by simply slowing the infusion rate. This patient had no other adverse effects with the infusion nor with her prior infusion.

Two patients had serious adverse effects. Both patients had nausea and vomiting associated with their first and second infusions (Day 1 & 8). They tolerated their test dose of 17-1A on Day 15 without adverse effects over 30 minutes of observation. The treatment infusions were then begun and both developed dyspnea, tachycardia and hypotension judged to be an anaphylactic reaction. Both infusions were immediately stopped (less than 10% of dose given) and patients responded well to therapy with corti-costeroids, epinephrine and antihistamines. No patient in the study developed abnormalities of urinalysis, complete blood count, renal or hepatic function.

Pharmacokinetics

The serial plasma 17-1A levels on each patient were analyzed and found to fit will with a 1 compartment model of plasma disappearance. The results for peak plasma concentration, plasma half-life and area under the curve are summarized in Table 2.

TABLE 2

PHARMACOKINETICS 17-1A (400 mg) IN MAN

Prior Antibody	Peak Conc. (ug/ml)	Half-life (hours)	AUC (hrs-ug/ml)
None (n = 5)	139 ± 8	15 ± 2	3013 ± 175
One (n = 10)	141 ± 5	14 ± 1	2828 ± 93
Two (n = 3)	108 ± 2	24 ± 2	3771 ± 81

Values are expressed as mean ± standard error of the mean.
AUC = area under the curve.

The two patients who had anaphylactic reactions did not receive their full third dose of 17-1A and therefore had no pharmacokinetic study. Thus, only 3 patients made up the group with two prior exposures to 17-1A. The results are similar for all 3 groups of patients. The three patients studied on their third infusion had a somewhat lower serum peak

concentration of 17-1A and a somewhat longer mean plasma half-life than the groups of patients with a single or second infusion. Interpretation is limited since the differences were modest and the group was made up of a small number of patients.

Human Anti-mouse Antibody (HAMA) Response

The patients' serum prior to therapy had little or no detectable ability to bind ^{125}I-17-1A coated beads. As summarized in Table 3, almost all patients developed HAMA within 29 days of their first 17-1A exposure (17/20). The majority (11/20) had HAMA by Day 8 with 8/11 having values of greater than 100 ng/ml and 2/11 having values of greater than 1000 ng/ml. Peak values were generally noted on Day 15 or 22 with values falling by day 29 and beyond. Patients who received one, two or three exposures to 17-1A had similar degrees by HAMA response as summarized in Table 4.

TABLE 3

HUMAN ANTI-MOUSE ANTIBODY (HAMA) RESPONSE*

Pre-therapy	0/20 had antibody (range - 0–15 ng/ml)
Day 8	11/20 had antibody (range - 36–106 ng/ml)
Day 15	15/20 had antibody (range - 27–5598 ng/ml)
Day 22	14/20 had antibody (range - 60–5046 ng/ml)
Day 29	15/20 had antibody (range - 23–4900 ng/ml)
No antibody	3/20

*Antibody activity expressed as ng of ^{125}I-17-1A bound/ml Plasma.

TABLE 4

DEGREE OF HUMAN ANTI-MOUSE ANTIBODY (HAMA) RESPONSE*

Exposures	Very High	(>1000)	Moderate	(40–999)	Poor/none	(<40)
Single	3	(60%)	1	(20%)	1	(20%)
Double	4	(40%)	4	(40%)	2	(20%)
Triple	2	(40%)	2	(40%)	1	(20%)

*Expressed as ng of 17-1A bound/ml plasma

The two patients with anaphylactic reactions were interesting. They has HAMA levels of 1055 and 264 ng/ml on Day 8 and 1716 and 3745 ng/ml on Day 15, respectively. They tolerated their infusions of antibody on Day 8 without adverse effect except for nausea and vomiting (similar to what they had on Day 1 infusion) but had anaphylactic reactions on Day 15 at the time of their third infusion. A total of 11 infusions were administered to patients when their HAMA levels were greater than 20 ng/ml (elevated) with five having no side effects, three gastrointestinal symptoms, one flushing/tachycardia and two anaphylactic reactions. No patients developed fever, proteinuria or renal impairment.

It was also interesting that in nine of these 11 infusions, adequate plasma samples were available to determine peak plasma concentration and plasma half-life of 17-1A antibody. These values were not substantially different than infusions in the absence of detectable HAMA.

Discussion

This phase I/II study of repeated administration of 400 mg 17-1A monoclonal antibody provides several observations. In general, the administration of antibody was well tolerated in patients receiving one or two infusions. The mild gastrointestinal symptoms were clearly related to antibody infusion and were not a serious clinical problem. The pathogenesis of these symptoms is not known but does not seem related to an allergic reaction since they occurred just as frequently during a patient's first infusion as compared to third infusion. They may be related to the ability of this antibody to bind to normal gastrointestinal mucosa (Sears, H., et al., *Surg. Res.* 31, 145–150 (1981)). Two of five patients receiving three weekly infusions of 17-1A had anaphylactic reactions. This frequency of a potentially lifethreatening allergic reaction precluded our testing a four-dose schedule (weekly) and would deter treatment schedules requiring antibody administration on Day 15.

The pharmacokinetic studies indicate that this dose of antibody can achieve plasma concentrations of 100–200 ug/ml with a plasma disappearance curve approximating observations with other mouse monoclonal antibodies (radiolabeled) administered at much lower doses (Pimm, M., et al., *J. Nucl. Med.* 26, 1011–1023 (1985) and Rosenblum, M., et al., *Cancer Res.* 45, 2382–2386 (1985)). This plasma half-life results in plasma concentrations of less than 1 ug/ml by day 8. Thus, maintenance of a substantial plasma concentration of 17-1A would require administration more frequently than weekly. Prior studies (Pimm, M., et al., *J. Nucl. Med.* 26, 1011–1023 (1985) and Larson, S., et al., *J. Nucl. Med.* 24:123-129 (1983)) have suggested that the appearance of HAMA response is associated with a dramatic alteration in circulating levels of mouse Ig. Our failure to observe this phenomenon is somewhat surprising. However, it should be noted that our antibody measurements are expressed in terms of ng 17-1A bound/ml plasma with an infusion of 17-1A which readily achieves concentrations of 100–200 ug/ml in the circulation. We are currently modifying our HAMA assay to allow quantitation of total circulating HAMA. This may clarify whether the patient's total circulating HAMA is able to bind only a small fraction of this large circulating dose of 17-1A.

We did not find evidence of pre-existing human anti-mouse antibody (17-1A) prior to antibody infusion as reported by others (Schroff, R., et al., *Cancer Res.* 45, 879–885 (1985)). We initially attempted to assay for human anti-mouse antibody using an assay which detected human immunoglobulin binding to 17-1A coated beads using radiolabeled monoclonal mouse anti-human Fc antibody. We found that normal individuals and cancer patients prior to monoclonal antibody infusion had varying quantities of human immunoglobulin which bound non-specifically to 17-1A coated beads. This binding did not have classic competitive inhibition by soluble antigen (17-1A) and was judged to be a non-specific phenomenon and not antibody. In contrast, post-immunization plasma immunoglobulin binding to 17-1A coated beads was readily inhibited by soluble antigen (17-1A). Thus, we believe that the double antigen assay system used in this study more clearly reflects immune response to 17-1A. Despite the administration of one or more large doses of 17-1A, human antibody response to this protein was prompt with antibody frequently detectable by Day 8 and appreciable levels of antibody achieved by Day 15 & 22. Further studies are underway to characterize this antibody response in regards to immunoglobulin subclass and anti-idiotype.

Biochemical and Epitope Analysis of 17-1A Antigen/Production of Monoclonal Antibodies Against 17-1A.

Materials and Methods

Cells and Tissues

The human colon carcinoma cell lines DLD-1 and WiDr were obtained form the American Type Culture Collection, Rockville, Md. The colon carcinoma line HT-29 was kindly provided by Dr. J. Fogh, Sloan Kettering Institute for Cancer Research, NY. Human tissues were snap-frozen in liquid nitrogen-cooled isopentane immediately after surgical removal.

Radiolabelling and Immunoprecipitation

Cells (5×10^7) were surface-labelled by lactoperoxidase-mediated iodination using ^{125}I as described. Pink, J. R. L. and Ziegler, A. (1979) in: *Research Methods in Immunology* Academic Press, N.Y. pp. 169–180. Immunosorbents prepared by binding MAbs to protein A-Sepharose (Sigma, St. Louis, Mo.) were added to labelled cell lysates at 4° C. for 2 hr. Bound material was eluted by boiling in sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli. Laemmli, U.K. (1970) *Nature*, 227: 680–685.

Selection of MAbs

Colon carcinoma tissue was obtained from a 51 year old female patient undergoing lobectomy of the liver because of a solitary metastasis. The 17-1A positive tumor tissue was carefully isolated, minced, homogenized and plasma membranes were purified as described by Touster et al. (1970) *J. Cell Biol.* 47 604–618. Fusion of the myeloma P3x63Ag8.653 and spleen cells from (C57BL/6 x Balb/c)F1 female mice was carried out using standard procedures (Galfre et al., (1977) *Nature* 266: 550–552) 3 days after a single i.p. injection of colon carcinoma plasma membrane preparations corresponding to 3 mg of protein together with Bordetella pertussis adjuvant. After fusion the cells were plated in HAT selective medium (hypoxanthine, aminopterin, thymidine) in 96-well microtiter plates containing mouse peritoneal macrophages. Supernatants of hybrids were screened by immunoperoxidase staining of frozen tissue sections derived from the liver metastasis taken for immunisation. Antibodies reacting with the colon carcinoma cells of the metastasis and the bile ducts in the adjacent liver, but not with hepatocytes, were further tested on a panel of non-malignant epithelial tissues listed in Table 5. Antibodies showing a 17-1A-like staining pattern in this context were cloned at least two times by limiting dilution.

TABLE 5

DISTRIBUTION OF 17-1A ANTIGEN IN NORMAL EPITHELIAL TISSUES[1]

Tissue	Number tested/ Number positive	Remarks
Colon	14/14	strong staining of mucosa
Small intestine	5/5	strong staining of mucosa
Stomach	6/9	occasional weak staining of restricted areas
Gall bladder	1/1	strong staining of mucosa
Pancreas	1/1	strong staining of acini, ducts and islets of Langerhans
Myer	6/6	strong staining of bile ducts; hepatocytes negative
Kidney	2/2	Loops of Henle moderately, distal tubules strongly stained; proximal tubules, glomeruli negative
Lung	2/2	strong staining of bronchi; alveoli moderately stained
Thyroid gland	3/3	strong staining of follicular epithlium
Mammary gland	3/3	strong staining of lobules and ducts
Thymus	0/1	
Skin	2/2	strong staining or sweat glands; epidermis negative

[1]as verified by immunohistochemical staining with MAbs 17-1A, M72, M74, M77 and M79, which showed a congruent staining pattern in all of the normal tissues examined.

Immunoblotting

Inmunoprecipitates were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes (Schleicher & Schull, Dassel, FRG) according to Towbin et al. (1979) *Proc. Natl. Acad. Sci.* USA 76: 4350–4354, and transferred antigen was visualized by an indirect immunoperoxidase technique, Holzmann, B. et al. (1985) *J. Exp. Med.* 161: 366–377.

Flow Cytometric Analysis

HT-29 cells were preincubated on ice with MAbs M72, M74, M77 or M79 as unconcentrated, 10×, or 50× concentrated supernatant, followed by incubation with biotinylated 17-1A antibody (10 ug/ml) and avidin-phycoerythrin (Becton-Dickinson, Mountain View, Calif.). The fluorescence profile was analyzed with an EPICS-V (Coulter Electronics, Hialeah, Fla.).

Immunohistochemistry

Frozen tissue sections were prepared and stained by an indirect immunoperoxidase technique essentially as described elsewhere. Gottlinger et al., (1985) *Int. J. Cancer* 35: 199–205. Briefly, air-dried sections (7 um) were fixed in acetone for 10 min., incubated with MAb (10 ug/ml or undiluted supernatant) for 30 min., washing in PBS and exposed for 30 min. to peroxidase-conjugated rabbit anti-mouse Ig antiserum (Dianova, Hamburg, FRG) diluted 1/200 in PBS containing 20% human serum. After washing in PBS the sections were incubated for 20 min. in 0.004% 3-amino-9-ethylcarbazole in 0.02 M barbital buffer, pH 7.4, containing 0.001% H_2O_2 and subsequently counterstained with Mayer's hemalum.

Results

Biochemical Analysis of the 17-1A Ag

Since the original biochemical analysis of the 17-1A Ag was only made for one human tumor cell line (HT-29), we investigated the nature of the 17-1A Ag expressed on two additional human colon carcinoma lines by surface iodination. Precipitation with MAb 17-1A revealed an identical single protein band in the three cell lines, DLD-1, WiDr and HT-29, which migrated with an apparent molecular weight of 37 kD in an SDS-PAGE system. As judged from fluorographic intensity, the amounts of antigen precipitable from the three cell lines were quite variable, with the colon carcinoma line DLD-1 providing the highest amount of radiolabelled antigen. Under reducing conditions, in which 2-mercaptoethanol was added to the precipitates prior to electrophoresis, a distinct band of 33 kD was obtained from all three cell lines. In addition, a major component of about 40 kD component was also found occasionally in HT-29 lysates. This band was apparently absent or could not be precipitated from WiDr cells.

Incubation of HT-29 cells with tunicamycin (2 ug/ml) for 24 hr resulted in the appearance of a new band of 30 kD under non-reducing and of 26 kD under reducing conditions, indicating that the 17-1A Ag contains 2 N-linked glycosylation sites. The glycoprotein nature of the 17-1A Ag was further substantiated by treatment of 17-1A precipitates with neuraminidase, which resulted in a slight but distinct reduction of the apparent molecular weight.

Epitope Analysis of the 17-1A Ag by new MAbs

Four new MAbs directed against the 17-1A Ag (M72, M74, M77, M79) were obtained by screening supernatants of hybridomas generated from mice immunized with membrane preparations from colon carcinoma metastases for a 17-1A-like reactivity on frozen tissue sections. All four antibodies precipitate proteins of identical molecular weight as seen with the original 17-1A antibody. In order to verify the identity of the antigens recognized by the new MAbs an extensive immunoblotting analysis was performed, whereby 17-1A immunoprecipitates were transferred to nitrocellulose paper after separation by SDS-PAGE and tested with the four new reagents. As shown previously, the four new

antibodies bound to a 37 kD protein was not obtained with isotype-matched control MABs. To analyze the epitope specificity of the four new MAbs cross-blocking experiments were carried out. In a flow cytometry analysis the binding of a biotinylated 17-1A antibody to HT-29 cells was completely blocked by preincubation of the tumor cells with MAbs M72 and M74 (Table 6). In contrast, MAbs M77 and M79 showed no significant blocking activity for the biotinylated 17-1A antibody at all concentrations tested. We further analyzed whether the epitope specificity could be related to the idiotypes of the antibodies. All MAbs were analyzed for reactivity with an anti-idiotypic antiserum generated against the 17-1A antibody in goats (kindly provided by Dr. Dorothy Herlyn, The Wistar Institute, Philadelphia). The anti-idotypic antiserum strongly reacted with the two cross-blocking MAbs (M72 and M74), but was completely unreactive with the MAbs M77 and M79 (Table 6). These data suggest that mAbs 17-1A, M72 and M74 recognize the same or closely related epitopes on the 37 kD glycoprotein, while MAbs M77 and M79 define additional epitopes on this antigen.

TABLE 6

IDIOTYPE EXPRESSION AND CROSS-BLOCKING ACTIVITY OF DIFFERENT MABS DIRECTED AGAINST THE 17-1A ANTIGEN

	17-1A	M72	M74	M77	M79
Reactivity with goat anti-Id 17-1A	+[a]	++	+	-	-
Cross-blocking activity	+[b]	++	++	-	-

[a]in an ELISA, where the MAbs were coated to microtiter plates and subsequent binding of the goat anti-idiotypic antiserum to the MAbs was revealed by an indirect immunoperoxidase technique.
+optical density (OD) > 0.8;
++OD > 1.6;
-OD < 0.05
[b]Inhibition of binding or biotinylated 17-1A antibody tested in a flow cytometric analysis (see Materials and Methods):
+>25%,
++80%,
-<10%

Tissue Distribution of the 17-1A Ag

Using an indirect immunoperoxidase technique the 17-1A Ag was identified in various normal human organs and in a variety of human carcinomas. Concomitantly the four new MAbs were analyzed on parallel tissue sections. With respect to expression of the 17-1A Ag on colon tissue, it was found that normal mucosa was stained to a similar degree as colon carcinoma tissue in the fourteen patients examined. The 17-1A Ag was clearly detectable on the epithelial lining of the small intestine, the gall bladder, the bronchi and a variety of glandular structures, including the tyroid, the mammary gland, sweat glands and the exocrine as well as endocraine pancreas. Furthermore, the 17-1A Ag was found to be expressed in the kidney on distal tubules and the loops of Henle and in the liver by bile ducts, but not by hepatocytes.

In the stomach, normal mucosa usually showed a faint staining restricted to defined areas. However, in five patients with various degrees of intestinal metaplasia, present in an islet-like fashion in the gastric mucosa, those lesions were strongly stained by MAb 17-1A and the four new MAbs. The 17-1A Ag was also distinctly expressed in 9 of 9 gastric carcinomas tested.

Discussion

We have demonstrated that the 17-1A Ag is a glycoprotein migrating in SDS-PAGE with an apparent molecular weight of 37 kD under non-reducing conditions. Comparative analysis of three different colon carcinoma lines did not indicate a hetereogeneity of the 17-1A Ag when non-reducing conditions were applied. After reduction of the 17-1A precipitates with 2-mercaptoethanol and separation by SDS-PAGE a 33 kD band appeared which could be found with all the cell lines tested. In addition, a 40 kD band could be seen as a major component in the DLD-1 cell lysate and to a lesser degree in the lysate of HT-29 cells. This 40 kD band was virtually absent in lysates from WiDr cells. When precipitates from DLD-1 or HT-29 cells were processed in single experiments and analyzed in a parallel fashion under reducing and non-reducing conditions, again two bands of 33 and 40 kD appeared in the presence, but only a single band of 37 kD in the absence of 2-mercaptoethanol. Thus, reduction of the 17-1A Ag may result in the formation of two new forms of the molecule with different electrophoretic mobilities. Most probably the presence of intrachain disulfide bonds may account for this peculiar migration behavior. Alternatively, the 17-1A Ag may actually exist as a dimer of proteins exhibiting the same migration behavior under non-reducing conditions. The absence of the 40 kD molecule in WiDr lysates may then be explained by a differential accessability of this protein for lactoperoxidase-mediated iodination in different cell lines. Further analysis using metabolic labelling and cross-linking chemicals may be required to solve this question. Ross et al. have recently described a carcinoma-associated surface glycoprotein defined by their MAb GA733, that consists of 30 kD and 40 kD subunits. They suggest that the 17-1A antibody recognizes the same antigen, but binds to a different epitope than MAb GA733. Direct comparisons with the antibodies described here should reveal the relationship of these antigens.

The four new anti-17-1A Ag MAbs which we obtained displayed a tissue reactivity comparable to the original 17-1A antibody in normal organs and in tumors. So far no differential epitope expression was found in a variety of tissues examined. Two of these MAbs (M72 and M74) recognize a determinant closely related to that detected by MAb 17-1A as judged by cross-blocking experiments, whereas the MAbs M77 and M79 apparently define at least one additional epitope since they did not inhibit binding of the biotinylated 17-1A antibody. Interestingly, the epitope specificity of these MAbs was correlated with their reactivity with a goat anti-idiotypic antiserum. These MAbs will be of interest for analyzing their biological activities in conjunction with the original 17-1A antibody.

The 17-1A Ag is broadly expressed in non-malignant epithelial organs and is also present in most carcinomas originating from 17-1A-positive tissues. Quantitative differences in expression between normal and malignant tissue were not evident in a comparative immunohistochemical analysis of colorectal carcinomas and normal mucosa obtained from the same patients. However, a possible structural heterogeneity of this epithelial antigen on normal and malignant cells is not ruled out by the present analysis.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A method for treating a gastrointestinal tumor comprising administering to a patient afflicted with a gastrointestinal tumor, a murine monoclonal antibody which specifically binds to an epitope of 17-1A antigen, said antibody

being administered in multiple doses of about 400 milligrams or more per dose.

2. The method of claim 1 wherein the antibody is administered in multiple doses of about 400 to 1,000 milligrams for a total of about 1 to 5 grams of antibody.

3. The method of claim 1 wherein the gastrointestinal tumor is gastrointestinal adenocarcinoma, colorectal or pancreatic carcinoma.

4. The method of claim 3 wherein the multiple high dose therapy is administered as adjuvant therapy.

5. A method for treating a gastrointestinal tumor comprising administering to a patient afflicted with a gastrointestinal tumor, a murine monoclonal antibody which specifically binds to an epitope of 17-1A antigen, said antibody being administered in multiple doses of about 100 milligrams or more per dose for a total of about 0.1 to 5 grams of antibody, wherein the multiple doses are administered such that the patient sustains a high plasma level of antibody during the therapy.

6. The method of claim 5 wherein the multiple doses are administered at an interval between about one day to about one week.

7. The method of claim 6 wherein the gastrointestinal tumor is gastrointestinal adenocarcinoma, colorectal or pancreatic carcinoma.

8. A method for treating a gastrointestinal tumor comprising administering to a patient afflicted with a gastrointestinal tumor, a murine monoclonal antibody which specifically binds to an epitope of 17-1A antigen, said antibody being administered as adjuvant therapy in multiple doses of 100 milligrams or more per dose for a total of about 0.1 to 5 grams of antibody.

9. The method of claim 8 wherein th gastrointestinal tumor is gastrointestinal adenocarcinoma, colorectal or pancreatic carcinoma.

10. A method of claim 8 wherein the first dose of said antibody is of about 400 milligrams or more per dose.

11. A method of claim 8 wherein said antibody is administered as adjuvant therapy to surgery.

12. A method for treating a gastrointestinal tumor comprising administering to a patient afflicted with a gastrointestinal tumor, a murine monoclonal antibody which specifically binds to an epitope of 17-1A antigen, said antibody being administered in multiple doses, wherein the first dose is of about 400 milligrams or more per dose and the second or subsequent dose is of about 100 milligrams or more per dose, for a total of about 0.1 to 5 grams of antibody.

13. A method for treating a carcinoma originating from a 17-1A-positive tissue comprising administering to a patient afflicted with a carcinoma originating from a 17-1A-positive tissue, a monoclonal antibody which specifically binds to an epitope of 17-1A antigen, said antibody being administered in multiple doses of about 100 milligrams or more per dose, wherein the multiple doses are administered such that the patient sustains a high plasma level of antibody during therapy.

14. A method for treating a carcinoma originating from a 17-1A-positive tissue comprising administering to a patient afflicted with a carcinoma originating from a 17-1A-positive tissue, a monoclonal antibody which specifically binds to an epitope of 17-1A antigen, said antibody being administered in multiple doses, wherein the first dose is of about 400 milligrams or more per dose and the second or subsequent dose is of about 100 milligrams or more per dose.

15. A method for treating a carcinoma originating from a 17-1A-positive tissue comprising administering to a patient afflicted with a carcinoma originating from a 17-1A-positive tissue, a monoclonal antibody which specifically binds to an epitope of 17-1A antigen, said antibody being administered as adjuvant therapy in multiple doses of about 100 milligrams or more per dose.

16. A method for treating a carcinoma originating from bronchial tissue or tissue of glandular structure comprising administering to a patient afflicted with a carcinoma originating from bronchial tissue or tissue of glandular structure, a monoclonal antibody which specifically binds an epitope of 17-1A antigen, said antibody being administered in multiple doses for a total dose of about 0.1 to 5 grams.

17. A method of claim 16 wherein said antibody is administered as adjuvant therapy to surgery.

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